REMARKS

Applicants initially wish to thank Examiner Kosack for the telephonic interviews conducted on November 5, 2008, and November 10, 2008. During the interviews, the outstanding § 112 rejections, claim objections, and proposed amendments were discussed. Consequently, based on the interviews, Applicants submit this Amendment and Response.

Claim Amendments

Claims 1-55 are pending, of which claims 8-12, 15, 20-30, 35-42, 44, and 50 are withdrawn from further consideration by the Examiner as being drawn to a non-elected invention. Applicants have amended claims 4, 7, 13, 14, 16, 17, 31, 43, 45-49, 51, 53 and 54; and cancel without prejudice claims 1-3, 5, 6, 52 and 55.

More specifically, claim 4 has been amended to incorporate the limitations from claims 5 and 6, and to clarify the definitions of **[O]** and **Z**. Support for the amendments can be found in paragraphs [0114] and [0154], Fig. 8, and original claims 5 and 6 of the originally filed application as published under U.S. Patent Publication No. US 2006/0116422 ("published application").

Claim 14 has been rewritten as an independent claim by incorporating limitations from original claim 4. Support for the amendments can be found in paragraphs [0114] and [0154], Fig. 8, and original claim 4 of the published application.

Claim 16 has been amended to correct claim dependency and to clarify the definition of **[O]**. Support for the amendments can be found in paragraph [0114] of the published application.

Claim 43 has been rewritten as an independent claim.

Claim 45 has been amended to clarify the claimed diagnostic assay process. Support for the amendments can be found in paragraph [0162] of the published application.

Claims 7, 13, 31, 46-49, 51, 53 and 54 have been amended to correct claim dependency and/or to make minor editorial changes.

Applicants submit that no new matter is introduced by these amendments.

After entry of this amendment, claims 4, 7-51, 53 and 54 will be pending for examination, of which claims 1, 14 and 43 are independent claims.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1-7, 13-14, 16-19, 31-34, 45-49 and 51-55 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement.

Specifically, the Office action alleges that there are no definitions of "specifiers", "self-eliminating multiple release spacers", "single release 1,(4+2n) electronic cascade spacers", "cyclization elimination spacers", "spacer systems", and "reactive moieties" provided within the specification.

Applicants submit that the term "specifiers" is defined and described in paragraphs [0006], [0114] and [0140]-[0153] of the published application. The term "cyclization elimination spacers" is defined and described in paragraph [0125]-[0134] of the published application. Claim 4 as amended now incorporates the formulae provided in original claims 5 and 6 which further clarify the definitions of "self-eliminating multiple release spacers or spacer system" (**C**, **D**, **E**, and **F**) and "single release 1, (4+2n) electronic cascade spacers" (**W** and **X**). In addition, amended claim 4 now recites that **A** is an ω-amino aminocarbonyl cyclization spacer (e.g., as described in paragraph [0125] of the published application), and amended claims 4 and 14 now recite that **Z** is a therapeutic or diagnostic moiety (e.g., as described in paragraph [0154] of the published application).

Applicants submit that as amended, the generic formula in claim 4 as further defined by the formulae incorporated from original claims 5 and 6 is now sufficiently detailed to show that the Applicants were in possession of the full scope of the claimed invention at the time of filing. In addition, because claim 14 recites specific representative species of **W**, **X**, **C**, **D**, **E** and **F** which are fully supported by the originally filed specification, Applicants submit that they were in possession of the full scope of the invention of claim 14 at the time of filing.

Accordingly, Applicants respectfully request that the rejections under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 4-7 and 13-14 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. More specifically, the Office action alleges that no definition for **[O]** is provided in the claims or the specification.

As mentioned above, definition for **[O]** can be found in paragraph [0114] and Fig. 8 of the published application. However, without acquiescing to the rejection, Applicants have amended claims 4 and 14 to clarify the definition for **[O]**.

Claims 45-49 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being incomplete for omitting essential steps. Without acquiescing to the rejection, Applicants have amended claim 45 to clarify the claimed diagnostic assay process.

Claim 51 was rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. More specifically, the Office action alleges that neither the claims nor the specification contain a definition of the acronyms ADEPT, PDEPT, MDEPT, VDEPT, and GDEPT. Applicants submit that antibody-directed enzyme prodrug therapy (ADEPT), polymer-directed enzyme prodrug therapy (PDEPT) or macromolecular-directed enzyme prodrug therapy (MDEPT), virus-directed enzyme prodrug therapy (VDEPT) or gene-directed enzyme prodrug therapy (GDEPT) are within the knowledge of one skilled in the art, for example, as described in, respectively, Bagshawe, K.D. *Drug Dev. Res.* 1995, 34, 220; Satchi, R.; Connors, T.A.; Duncan, R. *B. J. Cancer,* 2001, 85, 1070; Huber, B. E.; Richards, C.A.; Krentisky, T.A. *Proc. Natl. Acad. Sci. USA,* 1991, 88, 8039; and Niculescu-Duvaz D.; Niculescu-Duvaz I.; Friedlos, F.; Martin J.; Spooner, R.; Davis L.; Marais, R.; Springer, C.J. *J. Med. Chem.*, 1998, 41, 5297; copies of each of which are attached hereto as supporting documents.

Accordingly, Applicants respectfully request that the rejections of claims 4-7, 13-14, 45-49, and 51 under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.

Claims 52 and 55 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Without acquiescing to the rejections, Applicants have canceled claims 52 and 55. Accordingly, Applicants submit that these rejections have been rendered moot.

Rejections under 35 U.S.C. § 101

Claim 52 is rejected under 35 U.S.C. § 101 as allegedly not having set forth any steps involved in the claimed recitation of a use, thereby resulting in a claim which is not a proper process claim under 35 U.S.C. § 101. Because claim 52 has been canceled, Applicants submit that the rejection has been rendered moot.

The Examiner is urged to telephone the undersigned attorney to discuss any remaining issues. Early and favorable actions are respectfully solicited.

Respectfully submitted,

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DRUG DEVELOPMENT RESEARCH 34:220–230 (1995)

Research Overview

Antibody-Directed Enzyme Prodrug Therapy: A Review

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology		Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Many of the limitations of conventional cytotoxic chemotherapy and of attempts to achieve selectivity using antibody vectors can be overcome by a 2 or 3 stage prodrug system. An antibody vectors to tumour sites an enzyme that is not normally present in human extracellular fluids. The tumour located enzyme activates a subsequently administered prodrug. As with other antibody based systems the pharmacokinetics and biodistribution of the antibody-enzyme conjugate are critical elements. In contrast to antibody drug conjugates and radiolabelled antibodies, an enzyme can be inactivated in non-tumour tissues, or subjected to rapid clearance, without toxic effects, which allows high tumour to normal tissue ratios to be achieved. Enzymes conjugated to antibodies increase the problem of immunogenicity and require either the use of immunosuppressive agents, or the development of non-immunogenic catalysts. A small scale pilot clinical trial has shown the general feasibility of the approach.

Key Words: enzymes, antibodies, tumor, prodrug

INTRODUCTION

For more than 40 years there has been an intensive search for agents that are selectively cytotoxic to the common cancers. Such agents have proved elusive. Genetic approaches promise much for the future but still face major problems. The restriction of cytotoxic agents to tumour sites would provide greater drug concentration x time (or area under the curve, AUC) than can be achieved with conventional cytotoxic drug administration and should limit drug resistance and diminish their short and long term effects on normal tissues including therapy induced malignancies.

PROBLEMS WITH ANTIBODY BASED THERAPEUTIC APPROACHES

Antibodies directed at tumour associated antigens have shown selective uptake by various cancers. Conventional antibodies have, however, shown only limited capacity as cytotoxic agents in their own right and they have therefore been conjugated to a variety of cytotoxic drugs, biotoxins, and radioisotopes [Davies and O'Neill, 1974; Baldwin and Byers, 1986; Moolten and Cooperbrand, 1970; Fitzgerald et al., 1987]. One limiting factor with drugs and biotoxins conjugated to antibodies is heterogeneity in the distribution of antigenic targets [Primus et al., 1983]. Since these conjugates must be internalised to be cytotoxic, cells which fail to express adequately the target antigen escape and such non-antigen expressing cells can be clonogenic. Moreover, the number of cytotoxic molecules that can be conjugated to an antibody without loss of antigen binding capacity is limited [Baldwin and Byers, 1986] so that it can be dif-

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ficult to deliver an effective dose of drug. That limitation may not apply to biotoxins, particularly if they can be directed selectively at tumour vasculature and such targeting also overcomes the limitation of poor tumour penetration by antibody conjugates [Thorpe, 1992].

Conjugates with radioisotopes can reduce the problem of heterogeneity in antigen expression through cross-fire effects on bystander cells [Begent et al., 1989]. But with radioisotopes the normal tissue dose received in the period before tumour localisation of the radiolabelled antibody limits the administered dose to a level that is only effective against the most radiosensitive tumours such as βcell lymphomas [Grillo-Lopez, 1994].

Attempts to generate cytotoxic agents from inactive precursors using enzymes thought to be present in excess in tumours were unsuccessful because few, if any, human tumours have a sufficiently specific enzyme repertoire [Connors, 1978].

ANTIBODY DIRECTED ENZYME PRODRUG THERAPY (ADEPT)

This concept utilises antibodies directed at tumour associated antigens to carry unique enzymes to tumour sites; after the antibody enzyme conjugate (AEC) is cleared from plasma and normal tissues, a prodrug, a substrate to the enzyme, is given [Bagshawe, 1987; Bagshawe et al., 1988; Senter et al., 1988]. The tumour located enzyme converts the prodrug to its cytotoxic derivative. The active drug is a small molecule which is generated within extracellular space and is able to diffuse through the tumour. Since antigen expression is not required for internalisation of the free drug a bystander cell effect is obtained. If the half-life (t 1/2) of the active drug is short enough, drug that diffuses out of the tumour should decay before reaching normal cell renewal tissues.

PRELIMINARY CONSIDERATIONS

In developing components for an ADEPT system several principles were clear from the outset. First it was evident that each component affected the choice of the other components in the system and therefore they had to be considered in toto. Second, previous studies with antibodies indicated that tumour uptake required a relatively high concentration to be maintained for several hours and that the clearance of antibodies from plasma tended to be slow [Rogers et al., 1986a,b]. Early studies with AEC showed that these behaved in a broadly similar fashion to radiolabelled antibodies [Bagshawe, 1989; Melton et al., 1990]. This highlighted a potential problem with the ADEPT approach because although a high plasma concentration of AEC favoured good tumour localisation [Bagshawe, 1989] a low, preferably zero concentration of the enzyme in plasma and non-tumour tissues is needed when giving the prodrug.

Clearance of an antibody, or AEC, from tumour sites is slower than clearance from plasma and other tissues [Boxer et al., 1992] so that tumour:non-tumour ratios improve with time from administration of the AEC [Yuan et al., 1991]. However, the concentration of enzyme at tumour sites must be such that the turnover of prodrug is sufficient to generate a lethal concentration of active drug.

There is, therefore, a critical time window in which prodrug can be given most advantageously. Further, if there is a significant concentration of enzyme in plasma and normal tissue when prodrug is given, the active drug will be formed in these tissues and will be dose limiting when present or transported to sensitive tissues, such as bone marrow.

To maximise the time window for safe administration of the prodrug it has been found necessary with some ADEPT systems to accelerate plasma clearance of the conjugate [Bagshawe, 1989; Sharma et al., 1990]. With these considerations in mind the individual components can be considered.

ANTIGENIC TARGET

It is well known that there are few, if any, well characteristed tumour specific antigens. Most are described as tumour associated antigens since they tend to be expressed by some normal tissues but usually in much lower concentration than in some cancers [Baldwin and Byers, 1986]. For therapeutic targeting it is obviously essential to exclude expression of a targeted antigen by normal tissues such as haemopoietic stem cells. Surprisingly, non-specificity in the distribution of targeted antigens, such as carcinoembryonic antigen (CEA) expression in normal interstitial mucosae, has not been identified as a cause of toxicity in antibody based therapies.

Another characteristic of most tumour associated antigens, that of heterogeneity in distribution within a tumour cell population, has already been mentioned. Although a relatively homogenous distribution of antigenic targets is advantageous for any antibody based therapy, it is not expected to be critical to an ADEPT approach since active drug generated in extracellullar space has the potential to diffuse through a tumour and to enter both antigen expressing and non-expressing cells [Bagshawe, 1987, 1989; Senter et al., 1988].

Much of the clinical and experimental experi-

ence with antibodies for diagnostic and therapeutic purposes has been obtained with monoclonal antibodies directed at antigens such as CEA [Gold and Freedman, 1965; Goldenberg et al., 1978; Mach et al., 1981]. These antigens may be present on the cell surface for a limited time span and are often secreted into the extracellular space. No firm concensus has emerged on the superiority of secreted or non-secreted antigens as targets. It can be argued that a long dwell time of an antibody conjugate at the cell surface, as might be expected with an antigen that is membrane bound, would be advantageous, but other considerations such as antigen density [Yuan et al., 1991; Capone et al., 1984], which may be high with some secreted antigens, may also be important. Studies in xenografted mice with an AEC in which the antibody was directed at human chorionic gonadotrophin (hCG) secreted by a drug resistant gestational choriocarcinoma (CC3) [Searle et al., 1981] showed that tumour localisation of antibodies and AEC occurred [Begent et al., 1980] even though there was a high concentration of the antigen present in the plasma [Bagshawe, 1989; Springer et al., 1991a]. The plasma antigen accelerated the clearance of the AEC through the formation of immune complexes, making it possible to give prodrug at an early time point. Similar observations were made on accelerated clearance in a xenograft model in which high plasma concentrations of epithelial membrane antigen occurred [Davidson et al., 1991].

Modulation of antigen expression and internalisation of AEC are generally undesirable in an ADEPT system because both could contribute to loss and/or inactivation of enzyme from extracellular space and contact with the prodrug substrate. Nevertheless, an antigen-AEC complex which internalises the enzyme in an active state could have a role in ADEPT if the enzyme requires a co-factor present in intracellular fluid but absent from extracellular fluids. For such a system to be effective, enough active drug needs to be produced in antigen positive cells to have a lethal bystander effect on antigen deficient cells.

Heterogeneity in antigen expression also poses the problem that non-marker expressing cells may give rise to antigen negative metastases. Enhanced effects have been claimed when two drug antibody conjugates were used together and directed at different target antigens expressed by the same targeted tumour [Rowland et al., 1994].

The advantage of antigenic targets that are membrane bound, such as C-erbβ2 on some breast carcinomas, which retain an AEC for a prolonged period, is suggested by a preliminary report [Eccles et al., 1994] that administration of the prodrug could be delayed until 12–13 days after giving the AEC. In this C-erbβ2 expressing breast carcinoma xenograft, residual plasma enzyme was very low at 12 days yet there was sufficient enzyme retained in the tumour to achieve eradication of the xenograft with a single dose of prodrug. Impressive results were also obtained with a F(ab') anti-CEA β lactamase conjugate and a vinblastine derivative cephalosporin prodrug. Good tumour localisation and low plasma levels were achieved allowing the prodrug to be given 24 h after the AEC and resulting in tumour eradication [Meyer et al., 1993].

There have been reports that a variety of agents can augment the expression of tumour associated antigens. These include interferons [Guadagni et al., 1988], butyrate [Chou et al., 1977], glucocorticoids [Wilson and Jawad, 1982], transforming growth factor B [Chakrabarty et al., 1988], and tumour necrosis factor (TNF) [Smyth et al., 1988]. If the augmented expression were restricted to tumours and augmented antibody binding could be demonstrated in vivo such an effect could be useful but evidence for this is limited to TNFα [Melton et al., 1993b]. Moreover, there is at least one report of TNFα causing antigenic modulation in breast and ovarian cells [Giacomini et al., 1992].

ANTIBODIES

Antibodies with high binding affinity are probably necessary in ADEPT applications although it is possible that very high binding affinity ($K = 10^{10}$ or more) may result in antibody trapping in the perivascular regions of tumours [Fujimori et al., 1990]. F(ab')2 fragments of antibody have given better tumour to normal tissue ratios than intact antibody [Harwood et al., 1987] and similar findings were obtained when the same intact antibody and F(ab')2 fragments were conjugated to enzymes. Smaller size favours more rapid plasma clearance and tumour penetration [Boxer et al., 1992; Yuan et al., 1991]. However, when an AEC clearance or inactivation system [Sharma et al., 1994a,b] is used, the advantage may lie with intact antibody which achieves a higher percentage of administered dose per gram of tumour [Sharma et al., 1990; Harwood et al., 1987; Pedley et al., 1993]. Bivalent antibodies may have better dwell times than univalent forms and it has been claimed that univalent antibodies limit internalisation [Glennie and Stevenson, 1982] but in vivo testing is always necessary. The ability to modify antigen binding sites and create single chain Fv fragments with higher affinity than the original antibody [Chester et al., 1994; Hawkins et al., 1992] may be used to improve conju-

TABLE 1. Reported Prodrug-Enzyme Systems

Enzyme	Prodrug	Reference
Carboxypeptidase G2	Benzoic acid mustards	Bagshawe et al. [1988] Springer et al. [1990]
	Aniline mustards	Davies et al. [1994]
	Phenol mustards	Springer et al. [1994]
Alkaline phosphatase	Etoposide phosphate	Senter et al. [1988]
	Mitomycin phosphate	Senter et al. [1989] Haisma et al. [1992a]
Beta-glucuronidase	p-Hydroxyaniline mustard-glucuronide	Roffler et al. [1991]
	Epirubicin-glucuronide	Haisma et al. [1992b] Mitaku et al. [1994]
Penicillin-V-amidase	Adriamycin-N phenoxyacetyl	Kerr et al. [1990]
Penicillin-G-amidase	N-(4'-hydroxyphenyl acetyl) palytoxin	Bignami et al. [1992]
	Doxorubicin and melphalan	Vrudhula et al. [1993]
Beta-lactamase	Nitrogen mustard-cephalosporin p-phenylenediamine	Alexander et al. [1991]
	Vinblastine derivative-cephalosporin, cephalosporin mustard	Meyer et al. [1993] Svensson et al. [1993]
Beta-glucosidase	Cyanophenylmethyl-beta-D-gluco-pyranosiduronic acid	Rowlandson-Busza et al. [1991]
Nitroreductase	5-(Azaridin-1-yl-)2,4 dinitrobenzamide	Knox et al. [1988] Somani and Wilman [1994]
Cytosine deaminase	5-Fluorocytosine	Wallace et al. [1994]
Carboxypeptidase A	Methotrexate-alanine	Haenseler et al. [1992]

gate localisation. Even full humanisation of murine antibodies may not eliminate anti-idiotype responses but with an AEC the immunogenicity of the antibody component is a lesser problem than that of the enzyme component of the conjugate. Factors which influence the penetration into tumours of antibodies and antibody conjugates are multiple and have been analysed extensively [Jain and Baxter, 1988; Cobb, 1989].

ENZYME

Whilst one enzyme may activate many different prodrugs each pair constitutes a close partnership. The optimum pH of the enzyme should be close to that of tumour extracellular fluid and the enzyme substrate interaction should demonstrate high efficiency. However, a low substrate turnover rate (Kcat) may limit activation by a low residual enzyme level in normal tissues. Enzymes that require cofactors are undesirable unless the cofactor can be easily and safely administered throughout prodrug administration or if the co-enzyme is present in intracellular fluid but absent from extra cellullar fluids (see Antigenic Target).

An enzyme that is present in normal body fluids and tissues has been used in some ADEPT studies [Senter et al., 1988; Haisma et al., 1992a] but it is difficult to see how a widely distributed enzyme such as alkaline phosphatase can offer any prospect of specificity.

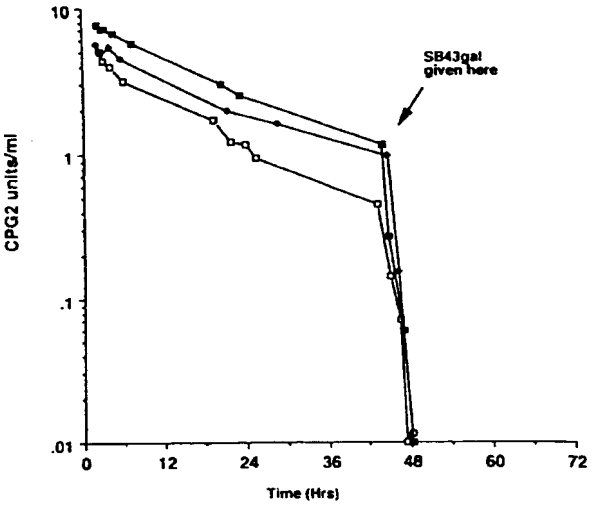
A non-mammalian enzyme such as carboxypeptidase G2 (CPG2) which catalyses the hydrolytic cleavage of L-glutamic acid from folates [Sherwood et al., 1985] has the advantage of having no human homologue and thus offers specificity limited only by the selectivity of the antibody delivery system. CPG2 like activity was found in the intestinal tract of the mouse [Antoniw et al., 1990; Springer et al., 1991b] but there was no evidence of comparable activity in man [Bagshawe, 1991]. The downside of such enzymes is their immunogenicity. To minimise the immunogenicity of antibody-enzyme conjugates, a human enzyme β-glucuronidase [Roffler et al., 1991; Haisma et al., 1992b; Mitaku et al., 1994] has been used but most studies have used non-human enzymes. These include penicillin V amidase [Kerr et al., 1990], penicillin G amidase [Bignami et al., 1992; Vrudhula et al., 1993], β glucosidase [Rowlandson-Busza et al., 1991], nitroreducatase [Knox et al., 1988; Somani and Wilman, 1994], carboxypeptidase A [Haenseler et al., 1992], and cytosine deaminase [Wallace et al., 1994] (Table 1).

The possibility of making catalytic antibodies [Iverson and Lerner, 1989] which could be humanised has particular relevance to ADEPT. However, the problems of achieving effective catalysts by this

route seem formidable; but phage technology [Chester et al., 1994; Hawkins et al., 1992], which facilitates the screening of large numbers of antibodies, may make it more feasible and, if so, humanisation of the targeting and catalytic components could greatly reduce the immunogenicity problem. Even so, it remains uncertain whether this would eliminate anti-idiotype responses [Schlueter et al., 1992]. Progress has also been reported in the production of synthetic enzymes which may also provide a basis for non-immunogenic catalysts [Kaiser, 1989].

The molecular size of the enzyme and hence of the conjugate has been considered by some authors to be important from the clearance aspect and, for example, clearance was rapid with a β-lactamase enzyme conjugated to a F(ab') anti-CEA antibody [Meyer et al., 1993]. Small size of the conjugate may also favour tumour penetration.

Up to the present time most work in the ADEPT field has been with antibodies or antibody fragments conjugated to enzymes by conventional heterobifunctional reagents [Melton et al., 1993a] but fusion proteins made by recombinant methods have also been reported [Bosslet et al., 1992] and large scale application will require recombinant products.



CLEARANCE OF CPG2 ACTIVITY FROM SERUM OF ADEPT PATIENTS GIVEN A5B7-F(ab')2-CPG2.

Figure 1. Clearance of antibody enzyme conjugate, measured as enzyme activity from serum of 3 patients who received anti-CEA $F(ab')_2$ conjugated to carboxypeptidase G2 over 48 h period. Infusion of galactosylated anti-enzyme antibody, SB43, started at 46 h.

CLEARANCE OF ANTIBODY ENZYME CLEARANCE

Clearance of the antibody-enzyme conjugate from non-tumour tissues whilst retaining an effective concentration at tumour sites constitutes a major issue in achieving the potential of the ADEPT approach. Effective partition of the enzyme's distribution between tumour and non-tumour tissues is essential. First it is necessary to achieve efficient localisation of the AEC at tumour sites. With an intact antibody or $F(ab')_2$ fragment as components of an AEC, efficient localisation at tumour sites requires a suitable concentration gradient from plasma to ECF to be maintained for several hours, typically 12–48 h. This requirement conflicts with the need to have low, preferably zero, enzyme activity in blood and normal tissues when prodrug is given.

Methods have been devised to reduce the plasma level of enzyme after tumour localisation has occurred. The AEC can be galactosylated so that it is quickly taken out of the circulation via hepatic galactose receptors [Sharma et al., 1990; Mattes, 1987; Ong et al., 1991]. By blocking those receptors temporarily with asialofetuin or bovine submaxillary gland mucoprotein (BSMP) the uptake of the galactosylated AEC is delayed allowing tumour localisation to occur. When the receptor blockade is terminated the galactosylated AEC is rapidly removed from blood via the hepatocytes. This method has been found effective in xenografted mice [Sharma et al., 1994b] but asialofetuin and BSMP are clearly unsuitable for clinical use and alternative galactose receptor blocking molecules are needed to pursue this option.

A method of accelerated clearance which has been tested clinically [Bagshawe, 1991] as well as in the mouse uses a second antibody (Fig. 1). A murine monoclonal antibody (SB43) inactivates the enzyme carboxypeptidase G2 (CPG2) and also accelerates the plasma clearance of the AEC [Sharma et al., 1990, 1991, 1994]. To prevent SB43 accessing and inactivating enzyme located in the tumour sites it is galactosylated (SB43-gal) so that it remains in the circulation long enough to bind to AEC and the immune complex is rapidly removed from the blood via hepatic galactose receptors. The fall in blood concentration of AEC (Fig. 1) may be followed by back diffusion of AEC from normal tissues into blood.

Improved tumour/blood conjugate ratios in a xenograft model have also been reported in a system using an antibody directed at cytosine deaminase [Kerr et al., 1993].

PRODRUG AND DRUG

Prodrugs can take various forms [Wilman, 1986]. One principle of prodrug development is the

introduction of an enzyme cleavable moiety onto a known cytotoxic drug. Drugs can be excluded from cells by cleavable phosphate groups or peptides [Senter et al., 1989]. Intercalating agents may be inactivated by cleavable steric groups [Roffler et al., 1991; Haisma et al., 1992] and electron withdrawing groups have been used to inactivate a range of alkylating agents [Springer et al., 1990, 1994; Davies et al., 1994]. A monofunctional alkylating agent has been converted to a difunctional agent by a nitroreductase [Rowlandson-Busza et al., 1991] (Table 1).

The toxicity differential between prodrug and drug is the dose limiting factor when all other elements in the system are controlled. It is also necessary for the active drug to be very reactive which is likely to be associated with a short half-life. However, the optimal t 1/2 is problematical in that if it is too short the bystander effect within a tumour mass may be limited. A t 1/2 for active drug of only a few seconds may be effective in a tumour where antigen distribution is relatively homogenous. The 15–20 min t 1/2 of the benzoic acid mustards used in the first pilot scale clinical study (see below) is undesirably long. Active drug in the vascular compartment is the cause of normal tissue toxicity and it has been suggested that it could be subjected to rapid deactivation by an enzyme restricted to the blood by conjugation to a suitable macromolecule [Bagshawe, 1994].

The ideal drug in the ADEPT context would not be subject to drug resistance. The fact that the relatively narrow range of resistance which occurs to alkylating agents [Frei et al., 1988] can be overcome by dose escalation argues for their use in a selective delivery system, but other potent cytotoxic agents have been at least as effective in xenograft models [Meyer et al., 1993].

SUMMARY OF EXPERIMENTAL STUDIES AT CHARING CROSS HOSPITAL

Published enzyme-prodrug systems are summarised in Table 1.

The development of a set of agents necessary for an ADEPT system is not a trivial undertaking and at the outset of studies at Charing Cross Hospital it was felt desirable to conduct feasibility studies in vitro, in vivo in xenografted mice, and if possible to take the same agents through to the clinic. Monoclonal antibodies directed at human chorionic gonadotrophin (hCG) and at carcinoembryonic antigen (CEA) were available and had been used extensively in clinical immunoscintigraphic studies. Carboxypeptidase G2 (CPG2) had been isolated from Pseudomonas and grown in *Escherichia coli* at the Centre for Applied

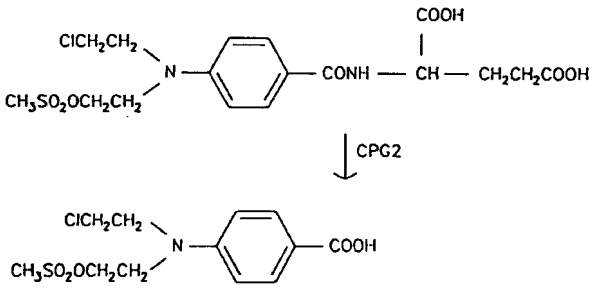


Figure 2. The glutamic acid cleaving action of carboxypeptidase G2 on the prodrug 4 [(2-chloroethyl) (2-mesyloxyethyl) amino] benzoyl-L-glutamic acid (CMDA).

Microbiology, Porton Down, Wilts, UK, and was made available to the study. Intact antibodies and F(ab')2 fragments were conjugated by thio-ether linkage and lyophilised. Samples were labelled with ^{131}I and tumour localisation in mice bearing choriocarcinoma or colorectal xenografts was shown by immunoscintigraphy [Bagshawe et al., 1988; Searle et al., 1981].

A series of benzoic acid mustards were converted to prodrugs by the introduction of an electron withdrawing glutamic acid moiety and it was shown that this was cleaved by CPG2 thus liberating the active mustard [Springer et al., 1990]. A bis-chloro benzoic acid mustard prodrug was first tested but a mono-mesyl, mono-chloro form 4-[(2 chloroethyl) (2 mesyloxyethyl) amino]-benzoyl-L-glutamic acid (CMDA) (Fig. 2) was found more effective both in vitro and in vivo. CMDA proved to have low in vitro toxicity such that an IC_{50} could not be determined. In the presence of CPG2 the cytotoxicity of CMDA on LS174T and JAR cells increased 50–100 times and was similar to that of the active drug [Antoniw et al., 1990; Springer et al., 1990].

In vivo studies with a choriocarcinoma xenograft (CC3) resistant to a variety of conventional cytotoxic agents showed that in most cases established tumours up to 1 cm were eliminated. The prodrug was given in 3 doses between 72 and 96 h post AEC [Bagshawe, 1989; Springer et al., 1991a].

The same protocol proved fatal to mice bearing LS174T colorectal carcinoma xenografts [Bagshawe, 1989]. The protocol was tolerated when the prodrug was delayed to 7 days post-AEC but then had little effect on tumour growth. The two xenograft models differed in that the CC3 tumours are associated with high plasma hCG which accelerates the clearance of AEC through immune complex formation and allows prodrug to be given 72 h post-AEC.

A monoclonal antibody SB43, raised to CPG2,

was found to inactivate CPG2 both in vitro and in vivo. When SB43 was given intravenously 24 h after CPG2 it sometimes resulted in the sudden death of the mice but in survivors there was a rapid reduction in CPG2 activity in blood. With unmodified SB43 there was a reduction in tumour located CPG2 activity. To avoid this SB43 was galactosylated (SB43-gal) which resulted in the antibody being taken up rapidly by hepatocytes but it still bound to CPG2 in blood and resulted in a rapid fall in plasma enzyme activity without a reduction in tumour located enzyme [Sharma et al., 1990, 1991].

SB43-gal given 19 h after 25–50 enzyme units of AEC caused the plasma enzyme level to fall promptly from >1 U/ml to <0.02 U/ml. It was necessary to give SB43-gal in 2 or 3 repeated small doses to avoid immune complex toxicity but with this 3 component system 20–25 days growth delay with the LS174T was obtained [Bagshawe et al., 1988].

Similar results have been obtained with established CEA producing ovarian carcinoma xenografts using the same antibody enzyme and prodrug [Sharma et al., 1994a].

An antibody directed at the conjugate which does not inactivate the enzyme and used as a second clearing antibody would result in accelerated clearance and may not require galactosylation.

PILOT SCALE CLINICAL TRIAL

F(ab')2 anti-CEA antibody (A5B7) conjugated to CPG2 has been used with SB43-gal and the CMDA prodrug in a small scale study in patients with advanced, drug resistant colorectal carcinomas [Bagshawe, 1991].

A preliminary study in 7 patients was used to test the toxicity of the prodrug alone. Administration was complicated by the drug being soluble only in dimethyl sulphoxide (DMSO). Even so, toxicity was minimal, consisting of nausea and occasional vomiting at total doses > 2 G/m^2. Studies with high pressure liquid chromatography (HPLC) gave no evidence of conversion of prodrug to its active product such as had been demonstrated in mice [Antoniw et al., 1990; Springer et al., 1991] and which was attributable to CPG2-like activity in murine intestinal contents. There was therefore no requirement for intestine sterilising antibiotics in patients.

The study with AEC showed that 20,000 enzyme U/m^2 of AEC (approximately 350 mg protein) gave plasma concentrations of 1-2U/ml 48 h post-AEC which were similar to those found optimal in the mouse models receiving the CMDA prodrug.

Initially SB43-gal was given by slow intravenous infusion over a minimum period of 3 h (Fig. 1). There was no morbidity attributable to the inactivating/clearing antibody administration. Doses of CMDA totalling >2 G/m^2 resulted in myelosuppression followed by return to normal haematological values within 25 days. The amount of SB43-gal given initially was 240 mg/m^2 but prolonging the duration of the SB43-gal infusion made it possible to reduce the dose to 50 mg/m^2. By the end of the study it was found that extending SB43-gal infusion at low dosage throughout the period of prodrug administration (Fig. 3) reduced or eliminated myelosuppression even when the total dosage of CMDA was increased to 10 G/m^2.

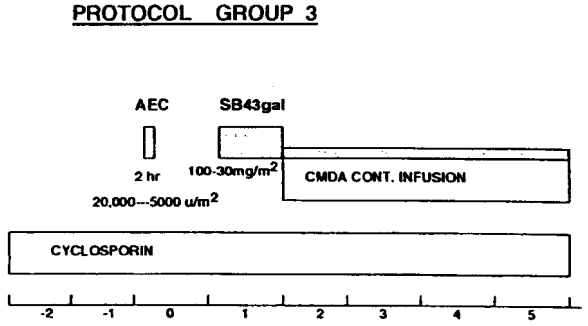


Figure 3. Protocol used for some patients in pilot scale clinical trial of ADEPT.

All patients developed antibodies to the mouse IgG and the bacterial enzyme CPG2 [Sharma et al., 1993]. Cyclosporin delayed the development of host antibodies (Fig. 4a and b) allowing up to 3 weekly cycles of therapy to be given [Bagshawe, 1991]. Two patients with severely impaired liver function who received cyclosporin orally died with hepatic and renal failure. Other patients received cyclosporin intravenously which facilitated better control of plasma cyclosporin levels. Of the 17 patients in this phase of the study 8 received a dosage of CMDA now judged to be potentially therapeutic (> 2.5 G/m^2). Of these 4 had partial remission and 1 had a mixed response where all but one liver metastasis regressed. Histological examination of the tissues removed at the initial laparotomy on the patient with a mixed response had shown both CEA positive and CEA negative lymph node metastases.

CONCLUSIONS

The feasibility of the ADEPT system has been demonstrated. Effective partition in the distribution of enzyme between tumour and non-tumour sites including blood will determine its ultimate success. Low levels of residual enzyme in normal tissues can

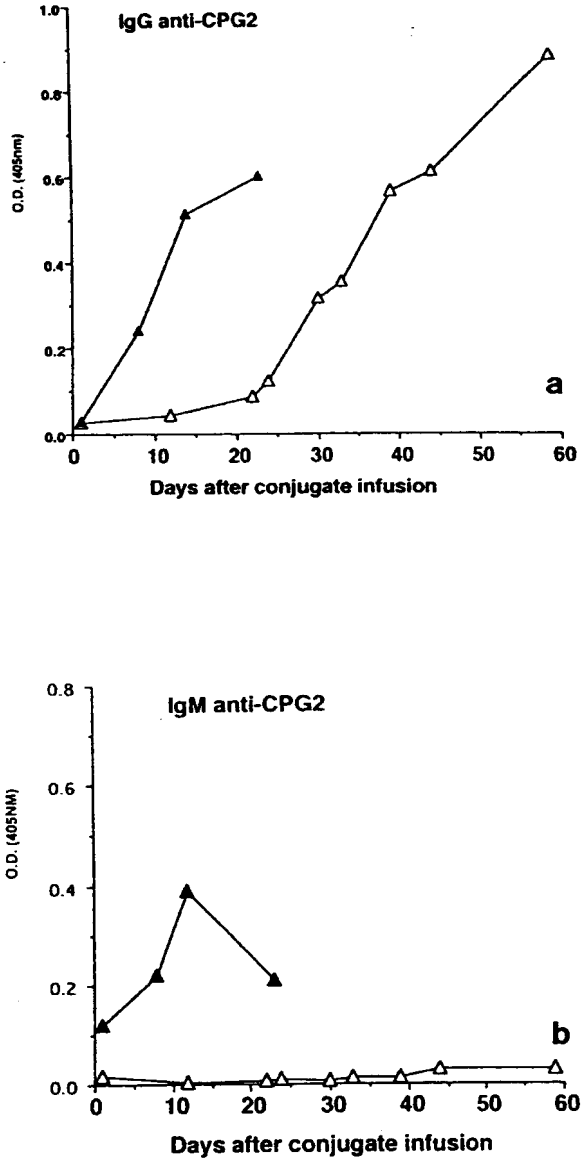


Figure 4. IgG (a) and IgM (b) responses of patients receiving antibody enzyme conjugate with (Δ-Δ) and without cyclosporin (▲-▲).

activate prodrug and are potentially dose limiting. A clearing mechanism with an antibody directed at the enzyme has proved effective both in the mouse and man. Clinical experience does not encourage the view that a single course of any therapy is likely to eradicate clinically evident carcinomas. Immunosuppression with cyclosporin has allowed up to three successive weekly cycles of treatment to be given but reducing or eliminating the immunogenicity of the antibody-enzyme conjugate would be advantageous. Efficacy has been demonstrated both in drug resistant xenograft models and in patients with advanced drug resistant colorectal cancer using a prodrug in the ADEPT mode.

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PDEPT: polymer-directed enzyme prodrug therapy

I. HPMA copolymer-cathepsin B and PK1 as a model combination

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Summary Polymer-directed enzyme prodrug therapy (PDEPT) is a novel two-step antitumour approach using a combination of a polymeric prodrug and polymer-enzyme conjugate to generate cytotoxic drug selectively at the tumour site. In this study the polymeric prodrug N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-Gly-Phe-Leu-Gly-doxorubicin conjugate PK1 (currently under Phase II clinical evaluation) was selected as the model prodrug, and HPMA copolymer-cathepsin B as a model for the activating enzyme conjugate. Following polymer conjugation (yield of 30–35%) HPMA copolymer-cathepsin B retained ~20–25% enzymatic activity in vitro. To investigate pharmacokinetics in vivo, ^{125}I-labelled HPMA copolymer-cathepsin B was administered intravenously (i.v.) to B16F10 tumour-bearing mice. HPMA copolymer-cathespin B exhibited a longer plasma half-life (free cathepsin B $t_{1/2\alpha}$ = 2.8 h; bound cathepsin B $t_{1/2\alpha}$ = 3.2 h) and a 4.2-fold increase in tumour accumulation compared to the free enzyme. When PK1 (10 mg kg^{-1} dox-equiv.) was injected i.v. into C57 mice bearing subcutaneously (s.c.) palpable B16F10 tumours followed after 5 h by HPMA copolymer-cathepsin B there was a rapid increase in the rate of dox release within the tumour (3.6-fold increase in the AUC compared to that seen for PK1 alone). When PK1 and the PDEPT combination were used to treat established B16F10 melanoma tumour (single dose; 10 mg kg^{-1} dox-equiv.), the antitumour activity (T/C%) seen for the combination PDEPT was 168% compared to 152% seen for PK1 alone, and 144% for free dox. Also, the PDEPT combination showed activity against a COR-L23 xenograft whereas PK1 did not. PDEPT has certain advantages compared to ADEPT and GDEPT. The relatively short plasma residence time of the polymeric prodrug allows subsequent administration of polymer-enzyme without fear of prodrug activation in the circulation and polymer-enzyme conjugates have reduced immunogenicity. This study proves the concept of PDEPT and further optimisation is warranted.

Keywords: PDEPT; tumour targeting; polymer prodrugs; HPMA copolymer; polymer-enzyme conjugate

Cancer chemotherapy is often restricted by adverse systemic toxicity and the appearance of multidrug resistance. Improvements, particularly for the treatment of common solid tumours, have been difficult to realise (Connors, 1996) and as a result combination therapies designed to increase active drug concentration in the tumour have been developed; namely antibody-directed enzyme prodrug therapy (ADEPT) (Bagshawe, 1987; Melton et al, 1996) and viral/gene-directed enzyme prodrug therapy (V/GDEPT) (Ram et al, 1993). Here we describe polymer-directed enzyme prodrug therapy (PDEPT), a novel two-step antitumour approach combining a polymeric prodrug and polymer-enzyme to generate cytotoxic drug at the tumour site (Figure 1). Conceptually, PDEPT proposes initial administration of the polymeric prodrug to promote tumour targetting before administration of the activating polymer-enzyme conjugate.

Polymer-enzyme conjugates such as polyethylene glycol (PEG)-L-asparaginase (Oncaspar®) have already been developed to the market (Ho et al, 1986). PEGylation of proteins reduces their immunogenicity and prolongs circulation time (Delgado et al, 1992). Although a new departure in cancer chemotherapy, several polymer-drug conjugates are already in early clinical trial (reviewed in Duncan, 1992; Duncan et al, 1996; Brocchini and Duncan, 1999). These include the N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer anticancer conjugates PK1 (FCE 28068) (Vasey et al, 1999), PK2 (FCE 28069) (Ferry et al, 1999) and PNU (166945) (Ten Bokkel Hunink et al, 1998) and a PEG-camptothecin conjugate (Pendri et al, 1998). Reduced toxicity and activity in chemotherapy refractory patients has been described. In phase I HPMA copolymer-doxorubicin (dox) (PK1) (Figure 2A) displayed a maximum tolerated dose of 320 mg/m^2 (Vasey et al, 1999) and also showed antitumour activity. Moreover the clinical pharmacokinetics (PK1 $t_{1/2\alpha}$ = 1.8 h with no dose dependency of clearance) were very similar to those reported in animals (Seymour et al, 1990). Combination of polymer-drug and polymer-enzyme conjugates can capitalise on the ability of both to target solid tumour tissue passively by a mechanism termed the enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986). This occurs due to the poorly organised tumour vasculature (Dvorak et al, 1988; Skinner et al, 1990) resulting in 'enhanced permeability' towards circulating molecules. The lack of lymphatic drainage in tumour tissue leads to increased 'retention'. The aim of this study was to establish the feasibility of the

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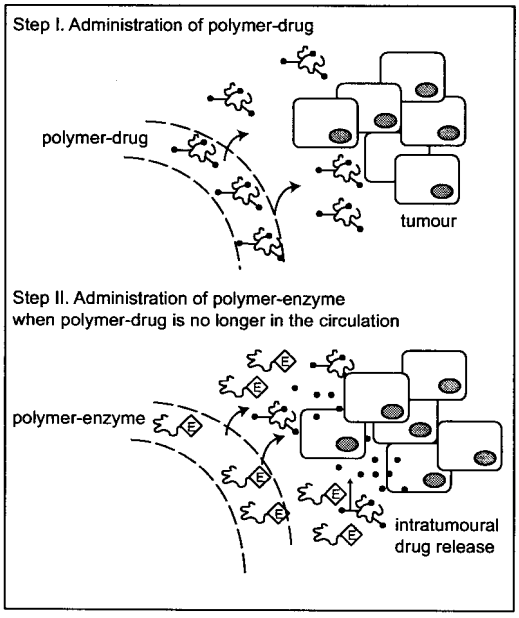

Figure 1 Schematic representation of PDEPT

PDEPT concept using PK1 and HPMA copolymer-cathepsin B as a model combination. PK1 has proven ability to target solid tumours by the EPR effect (Seymour et al, 1994) with concomitant renal elimination resulting in low blood levels within 1–5 h in animals and man (Seymour et al, 1990; Vasey et al, 1999). HPMA copolymer-cathepsin B (Figure 2B) was selected for PK1 activation as the PK1 Gly-Phe-Leu-Gly polymer-dox linker was designed to permit intralysosomal dox liberation due to action of the lysosomal cysteine proteases (Duncan et al, 1984). First it was necessary to prepare an HPMA copolymer-cathepsin B conjugate that would retain sufficient enzyme activity after conjugation. Activity was monitored in vitro using a low molecular weight substrate N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (Bz-Phe-Val-Arg-NAp) and the polymeric substrate PK1. The biodistribution of ^{125}I-labelled HPMA copolymer-cathepsin B and ^{125}I-labelled cathepsin B was assessed in mice bearing subcutaneous (s.c.) B16F10 tumours and this model was also used to measure the kinetics of doxorubicin release after intravenous (i.v.) administration of PK1 alone (drug liberation by endogenous lysosomal enzymes) or PK1 followed, after 5 h, by HPMA copolymer-cathepsin B. Preliminary studies were conducted to establish the antitumour activity of PDEPT combination using the B16F10 model and a human non-small-cell lung carcinoma xenograft (COR-L23).

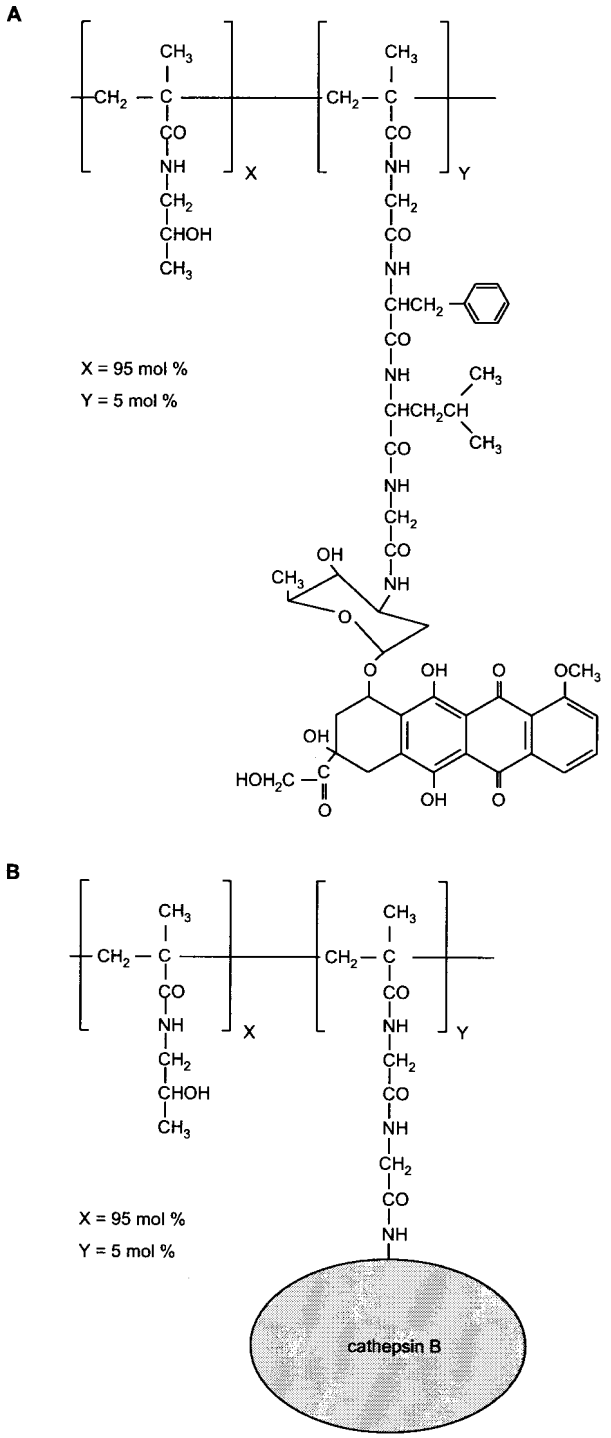

Figure 2 Structures of (**A**) PK1 and (**B**) HPMA copolymer-Gly-Gly-cathepsin B

MATERIALS AND METHODS

Materials

Cathepsin B (EC 3.4.22.1) from bovine spleen, reduced glutathione, Bz-Phe-Val-Arg-NAp, EDTA, Cu(II) sulphate pentahydrate 4% w/v solution, bicinchoninic acid solution, barbitone buffer (B6632) were all purchased from Sigma (Dorset, UK). Doxorubicin hydrochloride (dox) was obtained from Aldrich Chem (UK). Daunomycin hydrochloride (dnm) was kindly donated by Rhone Poulence (France). Tris-HCl 0.5 M, pH 6.8, Tris-HCl 1.5 M, pH 8.8, sodium dodecyl sulphate (SDS) 10% (w/v), TEMED, 10% (w/v) ammonium persulfate, mercaptoethanol, bromophenol blue and 30% (w/v) 19:1 acrylamide/bis-acrylamide were purchased from Bio-Rad (UK). PK1 was synthesised as previously described (Rihova et al, 1989) and batches had the following characteristics: M_w ~ 30,000 Da; M_w/M_n~1.3; 6–8% w/w of dox content. HPMA

copolymer-Gly-Gly-p-nitrophenol (ONp) was obtained from Polymer Laboratories (Church Stretton, UK).

The cell line B16F10 murine melanoma was kindly donated by Prof Ian Hart (St Thomas' Hospital, London, UK). COR-L23 non-small-cell lung carcinoma human xenograft cell line was purchased from ECCAC (European Collection of Cell Cultures, Centre for Applied Biology, Microbiology and Research, Salisbury, Wiltshire, UK). Bantin and Kingman Ltd (Hull, UK) supplied the C57 black male mice, Balb/C male mice and nu/nu male mice.

Synthesis and characterisation of HPMA copolymer-cathepsin B

Cathepsin B was bound to HPMA copolymer-Gly-Gly-ONp using a non-specific aminolysis reaction (Ulbrich et al, 1996). HPMA-Gly-Gly-ONp was dissolved in double distilled water (2 mg ml^{-1}) and the solution of cathepsin B (2 mg ml^{-1}) in 0.05 M phosphate buffer, pH 7.2, was added at 4°C with stirring. The reaction mixture was stirred in the dark at pH 7.2 for 30 min. Then the pH was carefully raised during 4 h by adding saturated sodium tetraborate buffer up to pH 8.5 (to prevent enzyme denaturation). The mixture was stirred for another 4 h and the reaction was terminated by adding 1-amino-2-propanol (1/2 the equivalent related to the original ONp content). The final yellow solution was acidified by adding diluted HCl solution to pH 7.2. The conjugation reaction was followed by UV spectrophotometry to detect the release of ONp with time from the HPMA copolymer precursor (bound ONp λ_{max} = 270 nm; free ONp λ_{max} = 400 nm). To remove free polymer, free enzyme and other low molecular weight compounds, the conjugate was purified at first by centriprep-50 (cut-off 50 KDa) or dialysis membrane Spectra/POR CE (Cellulose Ester) sterile DispoDialyzer (Pierce & Warriner) MW cut-off 50 KDa. The crude reaction mixture and the HPMA copolymer-cathepsin B preparations were subject to SDS PAGE analysis to ensure purity and the yield of the reaction determined by BCA protein assay.

Determination of cathepsin B activity

The in vitro activity of free and conjugated cathepsin B was first determined using the tripeptide substrate Bz-Phe-Val-Arg-NAp. Enzyme was incubated with substrate at 37°C in citrate phosphate buffer (0.2 M, pH 5.5) containing EDTA (10 mM) and reduced glutathione (50 mM). NAp release was followed spectrophotometrically at 410 nm. Subsequently the ability of free and conjugated enzyme to cleave the high molecular weight substrate PK1 was assessed by HPLC analysis. PK1 was incubated with cathepsin B at 37°C in a final volume of 1 ml: 400 μl of PK1 (1 mg ml^{-1}) in citrate buffer (pH 5.5, 0.2 M), 100 μl EDTA solution in buffer (10 mM), 100 μl reduced glutathione (GSH 50 mM) and 400 μl HPMA copolymer-cathepsin B or free cathepsin B in buffer (1 mg ml^{-1}). Samples (100 μl) were taken at various times, immediately frozen in liquid nitrogen and stored frozen in the dark until processed by HPLC (Wedge, 1990; Seymour et al, 1994) using daunomycin as internal standard (100 ng).

Tumour models

All animal experiments were carried out according to the United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare of animals in experimental neoplasia (UKCCCR guidelines, 1998).

Body distribution of free and conjugated ^{125}I-labelled-cathepsin B

Cathepsin B and HPMA-copolymer-Gly-Gly-cathepsin B were ^{125}I-radiolabelled with Na[^{125}I] iodide using chloramine-T. The labelling efficiency and purity (<1% free [^{125}I]iodide) of the ^{125}I-labelled products was then estimated using paper electrophoresis and the specific activity of each product was determined (μCi mg^{-1}). Male C57BL/6J mice were inoculated s.c. with 10^5 viable B16F10 cells. The tumour was allowed to establish until the area was approximately 50–70 mm^2 as measured by the product of 2 orthogonal diameters (*c.* 12 days). Free or conjugated cathepsin B (100 μl; 5×10^5 CPM) was injected into the tail vein of C57BL/6J mice (n = 3 per time point). The mice were then placed in metabolic cages and killed at times up to 48 h. Blood samples were taken and organs dissected, weighed and homogenised in PBS. Triplicate samples (1 ml) were then assayed for radioactivity. The results were expressed as % dose injected. The blood volume of the mouse was calculated assuming 5.7 ml blood/100 g mouse (Dreyer and Ray, 1910).

Evaluation of total and free doxorubicin after administration of PK1 and the PDEPT combination to mice bearing s.c. B16F10

Male C57BL/6J mice were inoculated s.c. with 10^5 viable B16F10 cells. Animals bearing palpable B16F10 were injected i.v. with PK1 (10 mg kg^{-1} dox-equiv.), or PK1 (10 mg kg^{-1} dox-equiv.) followed after 5 h by free cathepsin B (3.63 mg kg^{-1}) or HPMA copolymer-Gly-Gly-cathepsin B (3.63 mg kg^{-1} weight equiv. cathepsin B). They were killed at different time points up to 48 h post-PK1 injection. The following tissue samples were dissected: tumour, liver, kidneys, lung, heart, spleen, and urine and blood samples were collected. Samples were weighed, homogenised in PBS, mixed with 100 ng dnm. Total and free dox was analysed by HPLC (Wedge, 1990; Seymour et al, 1994).

Antitumour activity in B16F10 and COR-L23 models

Mice (6–8 weeks) were inoculated s.c. with tumour cells (10^5 B16F10 cells into C57 mice or 10^6 COR-L23 cells into nu/nu mice). When tumours became palpable (~25 mm^2) treatment was initiated. Groups (n = 5) were injected i.v. with PK1 (10 mg kg^{-1} dox-equiv.), or PK1 (10 mg kg^{-1} dox-equiv.) followed after 5 h, by free cathepsin B (3.63 mg kg^{-1}) or HPMA copolymer-Gly-Gly-cathepsin B (3.63 mg kg^{-1} weight equivalent of cathepsin B). Additional groups were treated with saline, free dox (10 mg kg^{-1}) or free cathepsin B (3.63 mg kg^{-1}). Animals were weighed and tumours measured daily. Mice were killed when the tumour reached or surpassed the size of 289 mm^2. The ratio (T/C) expressed as a percentage of the mean survival time for a treated group of animals (T) compared with the mean survival time of the control group (C) value was used to define antitumour activity. Statistical significance was assessed using the student's t-test for small samples and P values < 0.05 were taken as statistically significant.

RESULTS

HPMA copolymer-Gly-Gly-cathepsin B conjugate were prepared with a yield of 30–35% in respect of the bound protein. Whereas

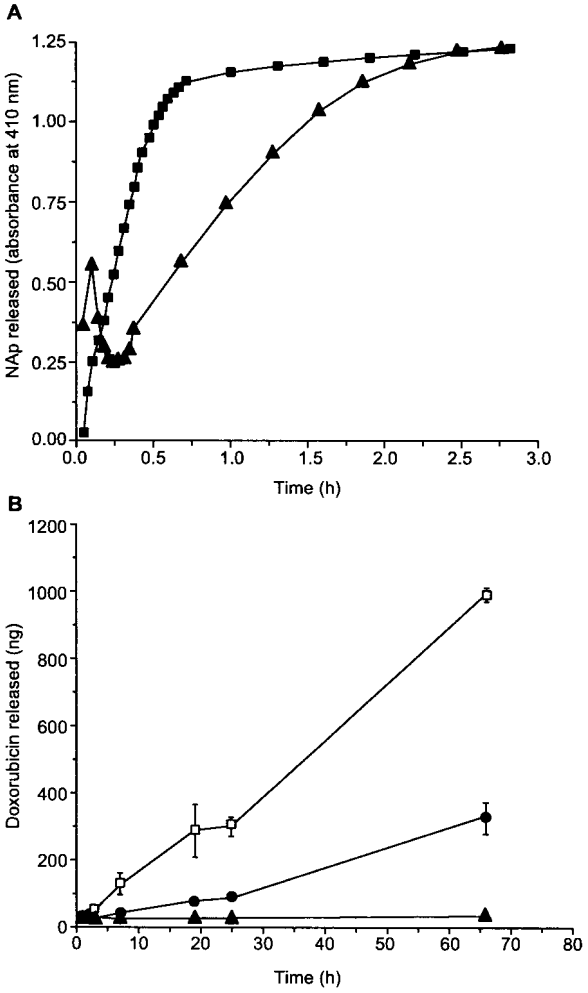

Figure 3 Comparison of enzymatic activity of free cathepsin B and HPMA copolymer-cathepsin B in vitro. Panel (**A**) Activity against Bz-Phe-Val-Arg-NAp; cathepsin B (■) and HPMA copolymer-cathepsin B (▲) and panel (**B**) using PK1 as a substrate; cathepsin B (□); HPMA copolymer-cathepsin B (●). Release of dox from PK1 in the absence of enzyme is included as a control (▲). See the Methods section for experimental details

free cathepsin B has a band at 30 kDa on SDS PAGE the HPMA copolymer-cathepsin B conjugates had a molecular weight in the range 60–97 kDa. After purification no free enzyme was detectable (results not shown). Against the Bz-Phe-Val-Arg-NAp substrate, HPMA copolymer-cathepsin B retained 24.4% the activity of free enzyme (Figure 3A) and with PK1 as substrate, the cathepsin B conjugate retained 19.5% enzyme activity (Figure 3B). The blood clearance of both the ^{125}I-labelled enzyme and conjugate was biphasic. Clearance of ^{125}I-labelled cathepsin B had a $t_{1/2\alpha}$ = 2.8 h and $t_{1/2\beta}$ = 8.9 h. The conjugate had a longer blood residence time ($t_{1/2\alpha}$ = 3.2 h and $t_{1/2\beta}$ = 9.3 h). The conjugate blood AUC was 1.3-fold greater than seen for free enzyme (Figure 4A) and the tumour AUC was 3.6-fold greater (Figure 4B).

After administration of PK1 to B16F10 melanoma-bearing mice the levels of free dox detected in tumour were constant with time at ~2500–5000 ng/tumour. When the HPMA copolymer-cathepsin B conjugate was administered 5 h after PK1 there was a marked increase in tumoural free dox levels (Figure 5). Over the 5–48 h period this caused a 3.6-fold increase in the free dox AUC

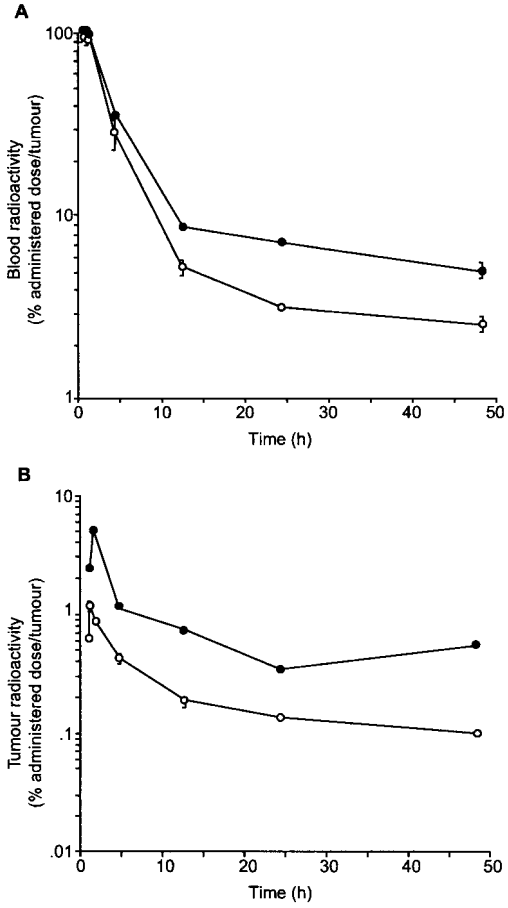

Figure 4 Body distribution of ^{125}I-labelled cathepsin B (○) and ^{125}I-labelled HPMA copolymer cathepsin B (●) in mice bearing s.c. B16F10 tumours. Panel (**A**) blood clearance of radioactivity and panel (**B**) tumour levels of radioactivity. Values represent the mean ± SE (n = 3)

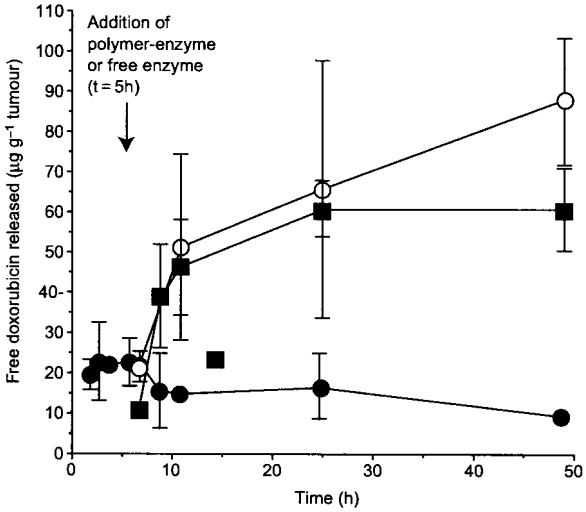

Figure 5 Levels of dox in B16F10 tumours after treatment with PK1 or the PDEPT combination. The data shown represent free doxorubicin levels after administration at time zero of PK1 (●), PK1 followed by HPMA copolymer-cathepsin B at 5 h (■) and PK1 followed by cathepsin B at 5 h (○). Whereas HPMA copolymer-cathepsin and free enzymes were given at the same enzyme dose (3.63 mg kg^{-1}) the free enzyme is ~ 5 × more active. Values represent the mean ± SE (n = 3)

Table 1 Antitumour activity of PK1 and the PDEPT model administered i.v. to C57 mice bearing s.c. B16F10 murine melanoma

Treatment	Dose (mg kg^{-1})	Time to progression (Days ± SE)	T/C (%)	No. of toxic deaths
Saline	–	6.8 ± 0.5	100	0/5
Doxorubicin	10	9.8 ± 0.9	144*	0/5
Cathepsin B	3.63	8.4 ± 1.4	123^{NS}	0/5
PK1 + cathepsin B	10	9.2 ± 0.8	135*	0/5
PK1	10	10.33 ± 0.3	152**	0/5
PK1 + HPMA-cathepsin B	10	11.4 ± 1.2	168**	0/5

Levels of significance * $P \leq 0.05$, ** $P \leq 0.01$, NS = no significant difference.

Table 2 Antitumour activity of PK1 and the PDEPT model administered i.v. to nu/nu mice bearing s.c. COR-L23 non-small cell lung carcinoma

Treatment	Dose (mg kg^{-1})	Time to progression (Days ± SE)	T/C (%)	No. of toxic deaths
Saline		19.6 ± 1.3	100	0/5
Doxorubicin	10	25.4 ± 1.6	129*	0/5
PK1	10	22.6 ± 1.4	115^{NS}	0/5
PK1 + HPMA-cathepsin B	10	24.8 ± 1.1	127*	0/5

Levels of significance * $P \leq 0.03$, NS = no significant difference.

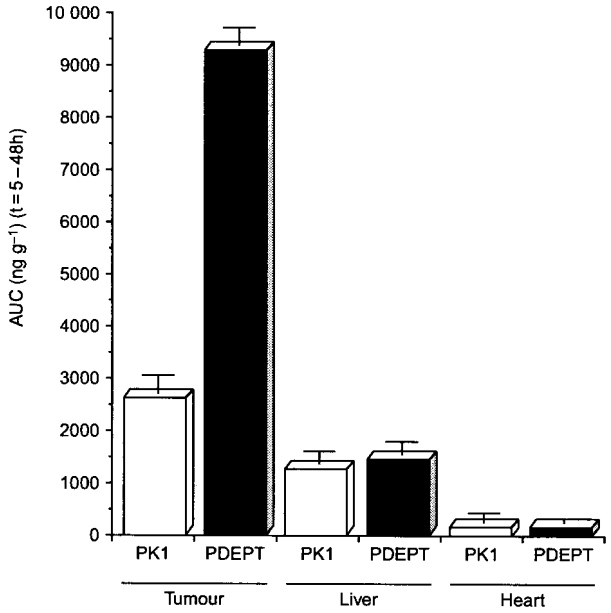

Figure 6 Free dox levels (AUC) measured in tumour and normal tissues after treatment of C57 mice bearing B16F10 s.c. tumours with either PK1 or the PDEPT combination

compared to that seen after administration of PK1 alone (Figure 5). Administration of free cathepsin B at the same protein dose (3.63 protein mg kg^{-1}; ~5 times the enzyme activity) resulted in an equivalent enhancement of dox liberation (results not shown). Little free dox (< less than 10 ng) was detectable in the blood at any time point (Figure 5) confirming that at 5 h after PK1 administration, no PK1 was available in the circulation to act as a substrate for the administered enzymes. Whereas, administration of HPMA copolymer-cathepsin B led to increased levels of free dox in tumour tissue there was no significant increase in dox levels in normal tissues (Figure 6).

A dox-equiv. dose of 10 mg kg^{-1} was chosen for all the treatments used in preliminary experiments investigating the antitumour activity of the PDEPT combination. Mice bearing s.c. B16F10 showed increased survival when treated with free dox, PK1 and the PDEPT combination (Table 1). Cathepsin B alone was not active. During this study there were neither toxic deaths nor animal weight loss (results not shown). A significant decrease (compared with control mice group) was observed in tumour growth rate in those animals treated with dox, PK1, and the PDEPT combination (Figure 7). Nude mice bearing COR-L23 showed increased survival when treated with dox alone and the PDEPT combination, although PK1 was not active in this model at the dose used (Table 2). A significant decrease in the tumour growth rate was observed after treatment of COR-L23 with either dox or the PDEPT combination (Figure 8).

DISCUSSION

Although ADEPT and V/GDEPT have demonstrated improved therapeutic activity in animal models and pilot human studies (Bagshawe et al, 1995; Springer and Niculesco-Duvaz, 1996), a number of inherent limitations have emerged. Antibody-enzyme conjugates are often highly immunogenic (Sharma et al, 1992) and individualised constructs are needed for tumours expressing different antigens. The long plasma half-life of antibody-enzyme leads to difficulties in optimisation of the dosing schedule for subsequently administered prodrug. In some cases a second clearing antibody has been required to prevent non-specific prodrug activation in the circulation. V/GDEPT allows combination of oncogene targeting with chemotherapy, but it has inherent problems associated with viral vectors, and the low and heterogeneous enzyme expression in vivo (Connors, 1995; McNeish et al, 1997). As the duration and reproducibility of enzyme expression is difficult to evaluate on an individual patient basis, optimisation of the schedule for prodrug administration again may be difficult. PDEPT offers a number of advantages in comparison with ADEPT and V/GDEPT HPMA copolymer-protein conjugates

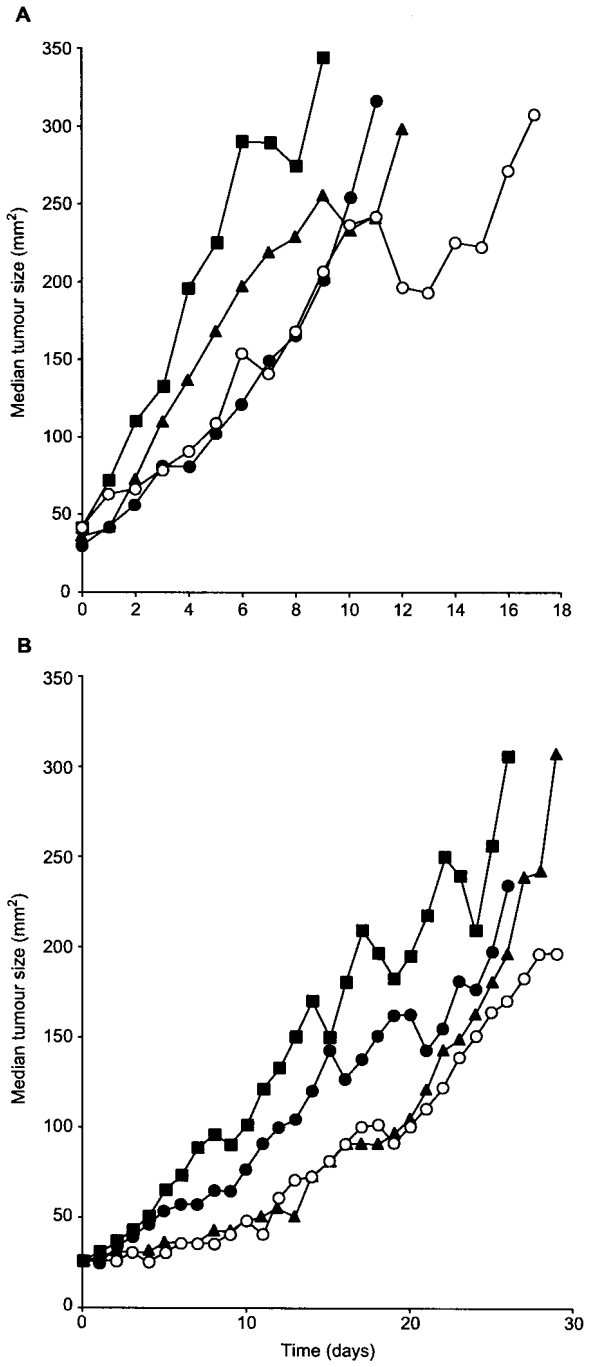

Figure 7 Tumour size after treatment with dox, PK1, cathepsin B or the PDEPT combination. Panel (**A**) shows B16F10 tumours and Panel (**B**) COR-L23 tumours. For both the key is saline (■), dox (10 mg kg^{-1}) (▲), PK1 (10 mg kg^{-1} dox-equiv.) (●) and PK1 (10 mg kg^{-1} dox-equiv.) + HPMA copolymer-cathepsin B (3.63 mg kg^{-1} protein-equiv) (○). Values represent the median tumour size

(like PEGylated proteins) display little or no immunogenicity (Flanagan et al, 1990). Additionally, the opportunity to administer a polymeric prodrug first (clinical pharmacokinetics has confirmed rapid plasma elimination whilst retaining the possibility of tumour targeting) can circumvent the problems associated with premature prodrug activation in the circulation.

Using the model combination of PK1 and HPMA copolymer-cathepsin B, this study has confirmed the feasibility of the 3 key aspects of the PDEPT concept. (1) Synthesis of a polymer-enzyme conjugate that retains activity against a polymeric prodrug, (2) tumour targeting of both components by the EPR effect and (3) spatial accessibility of polymer-enzyme to the polymeric prodrug in the tumour interstitium. Synthesis of antibody-and polymer-enzyme conjugates is often problematic. Conjugation typically has low yield (10–15%) (Svensson et al, 1994; Melton et al, 1996) and results in reduced enzyme activity. Ashihara et al (1978) showed that the activity of PEG-L-asparaginase conjugates decreased dramatically with increasing PEG molecular weight and in proportion to the degree of PEG substitution. The semi-random aminolysis method used to bind HPMA copolymer precursor to cathepsin B resulted in conjugates with a high yield (30–35% protein conjugation) and also conjugates with retained enzyme activity in vitro against both the low molecular weight and macromolecular substrates (20–25%) (Figure 3).

^{125}I-Labelled cathepsin B had a longer half-life ($t_{1/2\alpha}$ = 2.8 h) anticipated from the values reported for other (mainly bacterial) enzymes. This may be explained by the homology of the bovine and the mouse cathepsin B. None the less, the ^{125}I-labelled HPMA copolymer-cathepsin B conjugate did show a longer blood circulation (Figure 4A) consistent with the reduced cellular clearance and/or increased resistance to proteolysis (Francis et al, 1992). Increased circulation time led to improved conjugate tumour probably attributable to passive targeting by the EPR effect.

Using the B16F10 model, Seymour et al (1994) showed that i.v. administration of PK1 produced a total doxorubicin AUC that was ~17-fold higher than seen for free dox at equi-dose (5 mg kg^{-1}). It was also noted that dox liberation had a lag phase of 30–60 min and thereafter the free doxorubicin levels plateaued. The same basic pattern of PK1 doxorubicin release was observed here (Figure 5). However, subsequent administration of HPMA copolymer-cathepsin B (Figure 5) clearly led to a marked increase in PK1 doxorubicin liberation. This observation confirms both extracellular accessibility of the polymer enzyme to the polymer prodrug and verifies retention of the conjugate enzyme activity in vivo. Administration of free cathepsin B (the same protein dose but ~5 times the enzyme activity) also led to intratumoural release of doxorubicin from PK1 (Figure 5). The apparently greater potency of the polymer-enzyme conjugate in this experiment was simply consistent with the improved EPR-mediated targeting of the conjugate compared with to free enzyme.

It was considered important to ensure that the PDEPT combination would produce antitumour activity. Although the preliminary studies reported here are limited – using a single treatment and the same doxorubicin dose as used for the pharmacokinetic studies (10 mg kg) – they verify activity of the PDEPT combination in both the B16F10 and COR-L23 tumour models. Previous studies using the ADEPT system in a CC3 human choriocarcinoma xenograft (Springer et al, 1991) clearly showed that antitumour activity improved dramatically following optimisation of both schedule and dose of the antibody-enzyme conjugate and the prodrug. Future experiments with the HPMA copolymer-cathepsin B and PK1 model combination will optimise both HPMA copolymer-enzyme and PK1 doses and also the length of the repeated cycle of administration. As we understand more of the clinical profile of PK1 in the ongoing phase II studies it may be clinically interesting to consider an activating enzymes as an adjunct to this therapy. However, other PDEPT combinations involving non-mammalian

enzymes are currently considered priority for development and these include an HPMA copolymer-β-lactamase and a related polymeric prodrug (Satchi et al, 1999).

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Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy

(liver cancer/6-methoxypurine arabinonucleoside/varicella-zoster virus/thymidine kinase)

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ABSTRACT **An approach involving retroviral-mediated gene therapy for the treatment of neoplastic disease is described. This therapeutic approach is called "virus-directed enzyme/prodrug therapy" (VDEPT). The VDEPT approach exploits the transcriptional differences between normal and neoplastic cells to achieve selective killing of neoplastic cells. We now describe development of the VDEPT approach for the treatment of hepatocellular carcinoma. Replication-defective, amphotrophic retroviruses were constructed containing a chimeric varicella-zoster virus thymidine kinase (VZV TK) gene that is transcriptionally regulated by either the hepatoma-associated α-fetoprotein or liver-associated albumin transcriptional regulatory sequences. Subsequent to retroviral infection, expression of VZV TK was limited to either α-fetoprotein- or albumin-positive cells, respectively. VZV TK metabolically activated the nontoxic prodrug 6-methoxypurine arabinonucleoside (araM), ultimately leading to the formation of the cytotoxic anabolite adenine arabinonucleoside triphosphate (araATP). Cells that selectively expressed VZV TK became selectively sensitive to araM due to the VZV TK-dependent anabolism of araM to araATP. Hence, these retroviral-delivered chimeric genes generated tissue-specific expression of VZV TK, tissue-specific anabolism of araM to araATP, and tissue-specific cytotoxicity due to araM exposure. By utilizing such retroviral vectors, araM was anabolized to araATP in hepatoma cells, producing a selective cytotoxic effect.**

Somatic cell gene therapy is a rapidly developing therapeutic approach for the treatment of human disease. The first clinical trials involving retroviral-mediated gene transfer and gene therapy are ongoing. These first trials are assessing the ability to correct adenosine deaminase deficiency in severe combined immunodeficiency disease (1) and to modulate tumor-infiltrating lymphocytes in cancer patients (2, 3).

We now describe an approach for the treatment of neoplastic disease involving retroviral-mediated gene therapy. We call this approach "virus-directed enzyme/prodrug therapy" (VDEPT). The VDEPT concept exploits the transcriptional differences between normal and neoplastic cells to selectively kill the cancer cells. An artificial chimeric gene is created that is composed of tissue-specific transcriptional regulatory sequences (TRSs; for review, see ref. 4) linked to the protein coding domain of a nonmammalian enzyme. The nonmammalian enzyme metabolically activates a nontoxic prodrug to a cytotoxic anabolite. If the TRSs are from a tumor-associated gene, such as the hepatoma-associated α-fetoprotein (AFP) gene, then the artificial gene will produce tumor-specific expression of the nonmammalian enzyme and, consequently, tumor-specific production of the cytotoxic anabolite. Our initial choice of activating enzyme developed from the recent observation that 6-methoxypurine arabinonucleoside [9-(β-D-arabinofuranosyl)-6-methoxy-9*H*-purine (araM); Fig. 1] is selectively toxic to varicella-zoster virus (VZV)-infected cells (5). This selectivity results from the fact that araM is a good substrate for VZV thymidine kinase (TK) but a poor substrate for any of the three major mammalian nucleoside kinases. Once monophosphorylated in a VZV-infected cell, araM monophosphate can be further anabolized by cellular enzymes to produce the cytotoxic anabolite, araATP (21) (Fig. 1). If VZV TK can be selectively expressed in tumor cells, then the cytotoxic anabolite, araATP, will be selectively formed in those tumor cells upon treatment with the prodrug araM. We now describe the development of this approach for the treatment of hepatocellular carcinoma (HCC), but it should be appreciated that this approach may be applicable to other tumor types as well.

Artificial, chimeric genes that generate either liver-specific or hepatoma-specific expression of VZV TK were created and placed into a replication-defective, amphotrophic retroviral shuttle vector for tissue delivery. Subsequent to viral infection, these chimeric genes generated tissue-specific expression of VZV TK, tissue-specific anabolism of araM to araATP, and tissue-specific cytotoxicity due to araM exposure.

MATERIALS AND METHODS

Materials. araM, [8-^{14}C]araM (49 Ci/mol; 1 Ci = 37 GBq), and α,α,α-trifluorothymidine (TFT) were synthesized at the Wellcome Research Laboratories (Research Triangle Park, NC) (5 and 6). Tritiation of araM ([G-^{3}H]araM; 16 Ci/mmol) was performed by Moravek Biochemicals (Brea, CA). The VZV TK gene (7), designated here as 22TK, was provided by J. Ostrove [National Institutes of Health (NIH), Bethesda, MD]. Plasmid 2335A-1 containing essential albumin (ALB) TRSs, which is equivalent to the NB construct (8), was obtained from R. Palmiter (University of Washington, Seattle, WA). Plasmid XM5 containing the N2 vector (9) was obtained from S. Karlsson (NIH). Human AFP TRSs were isolated from plasmid pAF5.1-CAT (10) provided by T. Tamaoki (University of Calgary, Calgary, Canada).

Cell Lines. PA317 (CRL 9078), Hep G2 (HB 8065), Hep 3B (Hep 3B2.1-7; HB 8064), H-4-II-E (CRL 1548), WiDr (CCL 218), SW480 (CCL 228), Detroit 551 (CCL 110), and MCF-7 (HTB 22) cells were obtained from the American Type Culture Collection. HuH7 cells were supplied by B. Mason (Fox Chase Cancer Center, Philadelphia); ψ-2 and NIH 3T3

Abbreviations: VDEPT, virus-directed enzyme/prodrug therapy; TRS, transcriptional regulatory sequence; AFP, α-fetoprotein; ALB, albumin; araM, 6-methoxypurine arabinonucleoside [9-(β-D-arabinofuranosyl)-6-methoxy-9*H*-purine]; araATP, adenine arabinonucleoside triphosphate; VZV, varicella-zoster virus; TK, thymidine kinase; HCC, hepatocellular carcinoma; TFT, α,α,α-trifluorothymidine; HAT, hypoxanthine/aminopterin/thymidine; LTR, long terminal repeat; PCA, perchloric acid.

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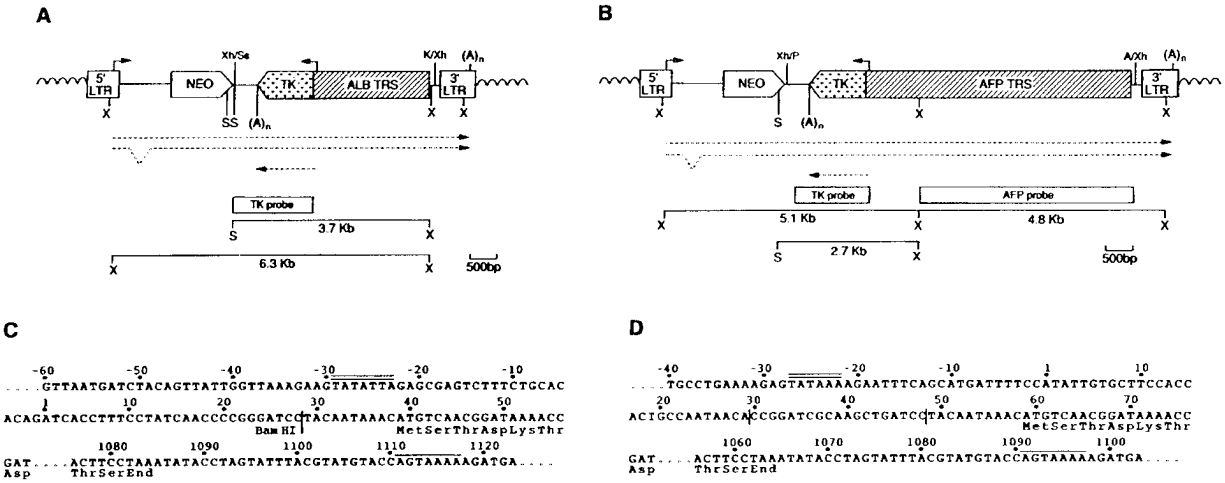

FIG. 2. Structure of VDEPT viruses CR74 and CR78. (*A* and *B*) Diagrams of pCR74 and pCR78, respectively. The VZV TK coding sequence is shown stippled; the ALB TRSs and AFP TRSs are shown hatched. Putative transcripts are shown as dashed lines. Hybridization probes and expected fragment sizes from Southern analysis are shown. ↱, Transcription initiation sites; $(A)_n$, poly(A) sites. A, *Aat* II; K, *Kpn* I; P, *Pst* I; S, *Sal* I; Ss, *Sst* I; X, *Xba* I; Xh, *Xho* I. (*C* and *D*) Partial DNA sequences of pCR74 and pCR78, respectively. Sequences are numbered with the start of RNA transcription as 1. The VZV TK coding sequence is translated [note: The entire VZV TK sequence is not shown since it has been published (17)]. The TATA box is double overlined, the poly(A)$^+$ signal is overlined. (*C*) Bases −60 to 28 are from ALB TRSs; bases 29–1123 are from VZV TK. (*D*) Bases −40 to 29 are from AFP TRSs; bases 30–48 are linker sequences; bases 49–1103 are from VZV TK.

orientation to eliminate non-tissue-specific expression of VZV TK from the 5′ LTR. Based on data from previous reports (18, 19), the putative transcripts generated from the recombinant retroviruses CR74 and CR78 are indicated in

Table 1. Hepatoma and nonhepatoma target cells

Cell line	Origin	ALB*	AFP*	Genetic alteration†	G418 resistant	VZV TK DNA	VZV TK activity‡	Cytotoxicity§ araA	Cytotoxicity§ araM
HepG2	Human liver	4100	2044	Parental	–	–	9	<1	>2000
				N2	+		4	<1	>2000
				CR74	+	+	4538		6.5
				CR74¶	+		6600		<0.5
				CR78	+	+	198		78
				CR78¶	+		1200		3.1
Hep 3B	Human liver	402	311	Parental	–	–	2	6	>2000
				CR74	+	+	44		367
				CR78	+	+	155		135
HuH7	Human liver	586	1842	Parental	–	–	13	3	1621
				CR74	+	+	2831		11
				CR78	+	+	200		36
H-4-II-E	Rat liver	ND (+++)‖	ND (+)‖	Parental	–	–	4	6	1848
				CR74	+	+	398		114
				CR78	+	+	26		160
MCF-7	Human breast	BT	BT	Parental	–	–	<1	<1	>2000
				CR74	+	+	<1		>2000
				CR78	+	+	14		1680
WiDr	Human colon	BT	BT	Parental	–	–	5	15.3	1028
				CR74	+	+	17		1751
				CR78	+	+	7		1744
SW480	Human colon	BT	BT	Parental	–	–	<1	<1	>2000
				CR74	+	+	<1		>2000
				CR78	+	+	14		1730
Detroit 551	Human skin	BT	BT	Parental	–	–	<1	<1	>2000
				CR74	+	+	<1		>2000
				CR78	+	+	18		1790

ND, not determined; BT, below threshold.

*Expressed as ng secreted per mg of cell protein per 4 days.

†Indicated for each cell line, parental unmodified cells (parental) or cells infected with the control virus (N2) (see ref. 13) or with the VDEPT viruses CR74 and CR78.

‡Expressed as pmol of araM phosphorylated per mg of protein per 30 min.

§Micromolar concentration producing a 50% decrease in DNA content after a 5-day exposure. araA cytotoxicity was determined in the presence of 5 μM *erythro*-9-(2-hydroxy-3-nonyl)adenine.

¶A particular clone of either Hep G2/CR74 or Hep G2/CR78 cell lines.

‖Major (+++) and minor (+) amounts of ALB or AFP synthesis based on historical data (20).

Fig. 2. DNA sequence analysis of pCR74 and pCR78 confirmed that the TATA boxes, the ALB or AFP TRS/VZV TK junction, and the entire VZV TK coding domain with its poly(A)$^+$ site were correct (Fig. 2 *C* and *D*).

Generation of Recombinant Retroviruses. Genomic DNA derived from clonal lines of PA317/pCR74 or PA317/pCR78 cells were isolated and analyzed by Southern blots to identify clonal lines containing a stably integrated, full-length retroviral vector. Two of the 15 PA317/pCR74 clonal cell lines analyzed contained only a full-length vector. Five of the 25 PA317/pCR78 clonal cell lines analyzed contained only a full-length vector.

Infection of Target Cells. Four hepatoma and four nonhepatoma cell lines were used as positive and negative target cells, respectively. AFP and ALB synthesis was measured for each human cell line (Table 1). Hep G2 cells have relatively high synthesis of AFP and ALB and Hep 3B cells have relatively low synthesis of both proteins, whereas HuH7 cells have relatively high AFP synthesis and moderate ALB synthesis. The rat hepatoma cell line H-4-II-E previously was shown to transcribe high levels of ALB (20). None of the human nonhepatoma cell lines had detectable AFP or ALB synthesis.

The full-length VDEPT viruses CR74 and CR78 were used to infect the hepatoma and nonhepatoma target cell lines to assess the tissue-specific expression of VZV TK. Subsequent to infection, cells were selected on G418 (Table 1). Genomic DNA was isolated and analyzed by PCR and DNA slot blots to confirm that all G418-resistant cell lines contained a stably integrated provirus containing a chimeric VZV TK gene (Table 1 and Fig. 3).

VZV TK enzymatic activity was determined in parental and CR74- and CR78-infected target cells. There was a barely detectable apparent rate of araM phosphorylation in cell extracts from control cells (parental) or cells infected with the control N2 virus. This low phosphorylation may result from the sequential action of adenosine deaminase and cellular kinases (Fig. 1) and was considered the background rate in this assay. Activity was increased in CR74- and CR78-infected hepatoma cells compared with the parental hepatoma cells (Table 1). VZV TK expression utilizing either the ALB or AFP TRSs roughly correlated to ALB or AFP production. VZV TK was not significantly increased in CR74- and CR78-infected nonhepatoma cells compared with the parental nonhepatoma cells (Table 1). These data indicate that the VDEPT viruses CR74 and CR78 were able to direct the tissue-specific expression of VZV TK, with expression being roughly correlated to ALB and AFP production.

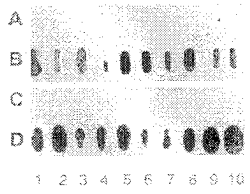

FIG. 3. Slot blot analysis of parental and infected cell lines. Rows A and B, amplified with 1 μM CR8 (CTCCTGCAGACTGGTACATACGTAAATACTAGG) and 1 μM CR9 (CTCGAGCTCGGTAAGTATGGTTAATGATCTACAG) specific for ALB TRS/VZV TK; rows C and D, amplified with 1 μM CR8 and 1 μM CR10 (CTCCGGTACCCCATTTTCAACCTAAGGAAATACC) specific for AFP TRS/VZV TK. A1–A9 and C1–C9, DNA from parental cells; B1–B9 and D1–D9, DNA from cells infected with CR74 and CR78, respectively. Slots: 1–9, H-4-II-E, HuH7, Hep 3B, Hep G2, WiDR, SW480, MCF-7, Detroit 551, and IMR90, respectively. B10 and D10, pCR74 and pCR78 plasmid DNA, respectively. A10 and C10, no DNA control.

Metabolism of araM. To confirm that Hep G2 cells expressing the VZV TK gene can anabolize araM to araATP, parental Hep G2 cells and Hep G2 cells infected with CR74 and CR78 were grown in the presence of [^{3}H]araM. After 48 hr, metabolites of araM were identified in cellular extracts by anion-exchange HPLC analysis. Fig. 4*A* illustrates that there were significant levels of araAMP, araADP, and araATP produced in CR74- and CR78-infected Hep G2 cells compared with Hep G2 parental cells. In addition, ADP and ATP were also labeled to higher specific activity, putatively through the action of cellular phosphatases on araIMP (Fig. 4; see Fig. 1). The levels of araAMP, araADP, and araATP were quantitated and are illustrated in Fig. 4*B*. There were just detectable levels of araATP produced in parental Hep G2 cells after 48 hr of incubation with araM. These low levels presumably were the result of adenosine deaminase activity, which may slowly convert araM to araH (see Fig. 1). These low levels of araATP did not produce any significant cytotoxicity in parental Hep G2 cells (see below). Compared with Hep G2 parental cells, there was an ≈7000-fold and ≈2000-fold increase in araATP levels in Hep G2 cells infected with CR74 and CR78 viruses, respectively.

AraM-Mediated Growth Inhibition. The data above suggest that the infected hepatoma cells that express VZV TK may now become selectively sensitive to agents that require VZV TK for efficient anabolism to cytotoxic metabolites. All cell lines experienced approximately equal growth-inhibitory effects resulting from araA exposure, indicating that if araATP was produced, these cells would exhibit a cytotoxic effect (Table 1). However, only the hepatoma cells that had signif-

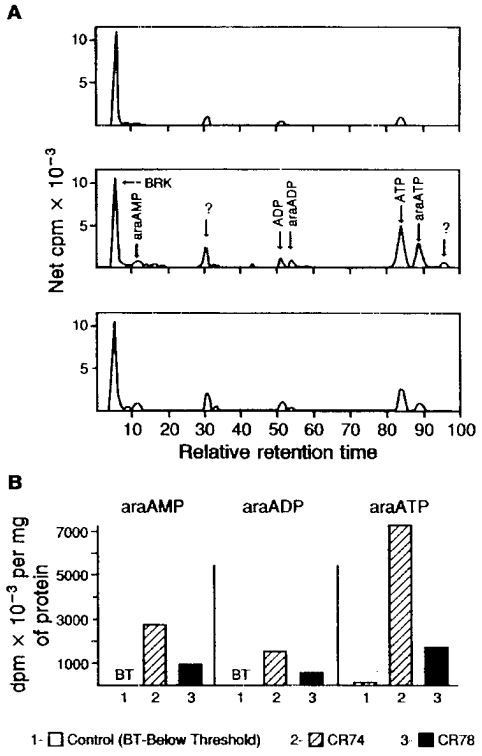

FIG. 4. Metabolism of araM. (*A*) Anabolism of araM in parental Hep G2 cells (control; top) and Hep G2 cells infected with CR74 (middle) and CR78 (bottom). BRK, breakthrough. (*B*) Relative anabolism of araM to araAMP, araADP, and araATP in parental Hep G2 cells (control) and Hep G2 cells infected with CR74 and CR78. Total recovery was based on the recovery of the internal standard, inosine triphosphate.

CO
G418
HAT
TFT
G418 + HAT
G418 + TFT
NIH 3T3
NIH 3T3/Tk⁻⁻
NIH 3T3/Tk⁻⁻/74-9
3/Tk⁻⁻/76.12
NIH 3

Self-Immolative Nitrogen Mustard Prodrugs for Suicide Gene Therapy

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Four new potential self-immolative prodrugs derived from phenol and aniline nitrogen mustards, four model compounds derived from their corresponding fluoroethyl analogues and two new self-immolative linkers were designed and synthesized for use in the suicide gene therapy termed GDEPT (gene-directed enzyme prodrug therapy). The self-immolative prodrugs were designed to be activated by the enzyme carboxypeptidase G2 (CPG2) releasing an active drug by a 1,6-elimination mechanism via an unstable intermediate. Thus, *N*-[(4-{[4-(bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenyl)carbamoyl]-L-glutamic acid (**23**), *N*-[(4-{[4-(bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamic acid (**30**), *N*-[(4-{[*N*-(4-{bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamic acid (**37**), and *N*-[(4-{[*N*-(4-{bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)-carbamoyl]-L-glutamic acid (**40**) were synthesized. They are bifunctional alkylating agents in which the activating effects of the phenolic hydroxyl or amino functions are masked through an oxycarbonyl or a carbamoyl bond to a benzylic spacer which is itself linked to a glutamic acid by an oxycarbonyl or a carbamoyl bond. The corresponding fluoroethyl compounds **25**, **32**, **42**, and **44** were also synthesized. The rationale was to obtain model compounds with greatly reduced alkylating abilities that would be much less reactive with nucleophiles compared to the corresponding chloroethyl derivatives. This enabled studies of these model compounds as substrates for CPG2, without incurring the rapid and complicated decomposition pathways of the chloroethyl derivatives. The prodrugs were designed to be activated to their corresponding phenol and aniline nitrogen mustard drugs by CPG2 for use in GDEPT. The synthesis of the analogous novel parent drugs (**21b, 51**) is also described. A colorectal cell line was engineered to express CPG2 tethered to the outer cell surface. The phenylenediamine compounds were found to behave as prodrugs, yielding IC_{50} prodrug/IC_{50} drug ratios between 20- and 33-fold (for **37** and **40**) and differentials of 12−14-fold between CPG2-expressing and control LacZ-expressing clones. The drugs released are up to 70-fold more potent than 4-[(2-chloroethyl)-(2-mesyloxyethyl)amino]benzoic acid that results from the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA) which has been used previously for GDEPT. These data demonstrate the viability of this strategy and indicate that self-immolative prodrugs can be synthesized to release potent mustard drugs selectively by cells expressing CPG2 tethered to the cell surface in GDEPT.

Introduction

The major objective in cancer treatment is to kill tumor cells selectively without harming normal cells.[1] A strategy for achieving selectivity is called suicide gene therapy. One approach aims to deliver toxin genes to the cancer cells.[2] An alternative approach has been named gene-directed enzyme prodrug therapy (GDEPT)[3] or virally-directed enzyme prodrug therapy (VDEPT).[4] These therapies consist of tumor-specific conversion of prodrugs to active drugs following the delivery of genes for exogenous enzymes.[5]

Self-immolative prodrugs have been proposed for activation by tumor enzymes.[6−8] A self-immolative prodrug can be defined as generating an unstable intermediate which, following the activation process, will extrude the active drug in a number of subsequent steps.[6,8] A cascade of events are required. First, the activation process, which is enzymatic, culminates in the extrusion process that generates the drug. The site of activation will usually be separated from the site of extrusion. The advantage of self-immolation is that it increases the diversity of prodrugs which can be activated by a certain enzyme.

Self-immolative mustard prodrugs for activation by *Escherichia coli* nitroreductase in a GDEPT system have previously been synthesized and evaluated in tumor cell lines.[9] An alternative candidate for GDEPT is carboxypeptidase G2 (CPG2) from *Pseudomonas* sp.[10] since it has no mammalian homologue.[11] This enzyme catalyzes the scission of an amidic,[11,12] urethanic, or ureidic[13,14] bond between an aromatic nucleus and L-glutamic acid.

The enzyme CPG2 has recently been mutated and expressed tethered to the outer cell membrane in the human breast carcinoma cell line MDA MB 361.[15] The expression of CPG2 tethered to the surface of the cell overcomes the need for a prodrug to penetrate the tumor cell membrane. Incubation of the transfected lines with the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]-

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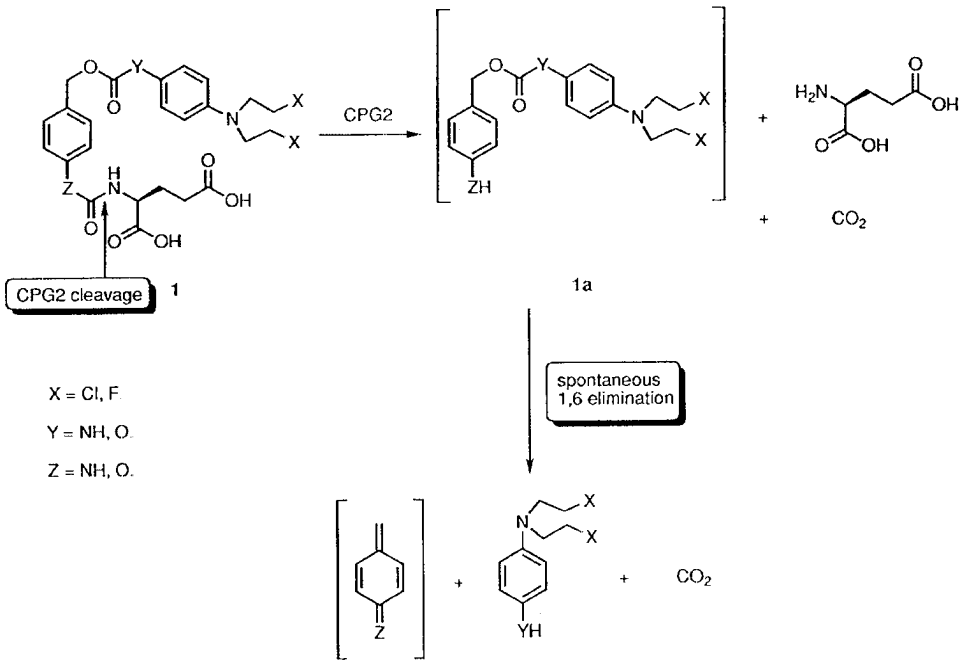
Scheme 1



benzoyl-L-glutamic acid (CMDA) leads to an increased sensitivity.[15] The prodrugs described herein were designed to release more potent active drugs than that cleaved from CMDA.

This report describes the first example of self-immolative prodrugs for activation by CPG2. It also describes the construction of the colorectal cell line LS174T made to express CPG2 tethered to the cell surface (LS174T-stCPG2(Q)3). Self-immolative prodrugs depicted as **1** in Scheme 1 were designed to be activated by CPG2 to generate the unstable intermediates **1a**, further releasing the active drug by a 1,6-elimination mechanism.

Rationale

For the synthesis of the novel nitrogen mustards prodrugs of general formula **1** (see Scheme 1), two self-immolative linkers: (4-hydroxymethylphenyl)carbamoyl-L-glutamic acid **12** and (4-hydroxymethylphenyl)-oxycarbonyl-L-glutamic acid **20** were designed, synthesized, and studied. The ureidic and carbamic linkages in **12** and **20**, respectively, between glutamic acid and the aromatic nucleus have a dual purpose. They provide a substrate cleavable by CPG2 and release an amino or hydroxy group required for the 1,6-elimination.

Aniline and phenol nitrogen mustards were chosen since the drugs are potent[16] and it is possible to reduce greatly their chemical reactivity through acylation. They were transformed to the corresponding self-immolative prodrugs **23**, **30**, **37**, and **40** by coupling with **12** or **20** through an oxycarbonyl or carbamoyl bond. The carbonate and carbamate bonds deactivate the phenol and aniline mustard, respectively by acylation, also acting as leaving groups in the 1,6-elimination, thus generating the drug via an irreversible process. The mechanism of activation of the prodrugs by CPG2 is presented in Scheme 1 and the physicochemical and kinetic data are in the Physicochemical and Kinetic Data section.

The corresponding fluorine mustard analogues **25**, **32**, **42**, and **44** were also synthesized. The rationale was to obtain compounds with greatly reduced alkylating abilities that would not react with nucleophiles as quickly as the corresponding chloroethyl derivatives. This enabled studies of these compounds as substrates for CPG2, without incurring the rapid and complicated decomposition pathways of the chloroethyl derivatives.

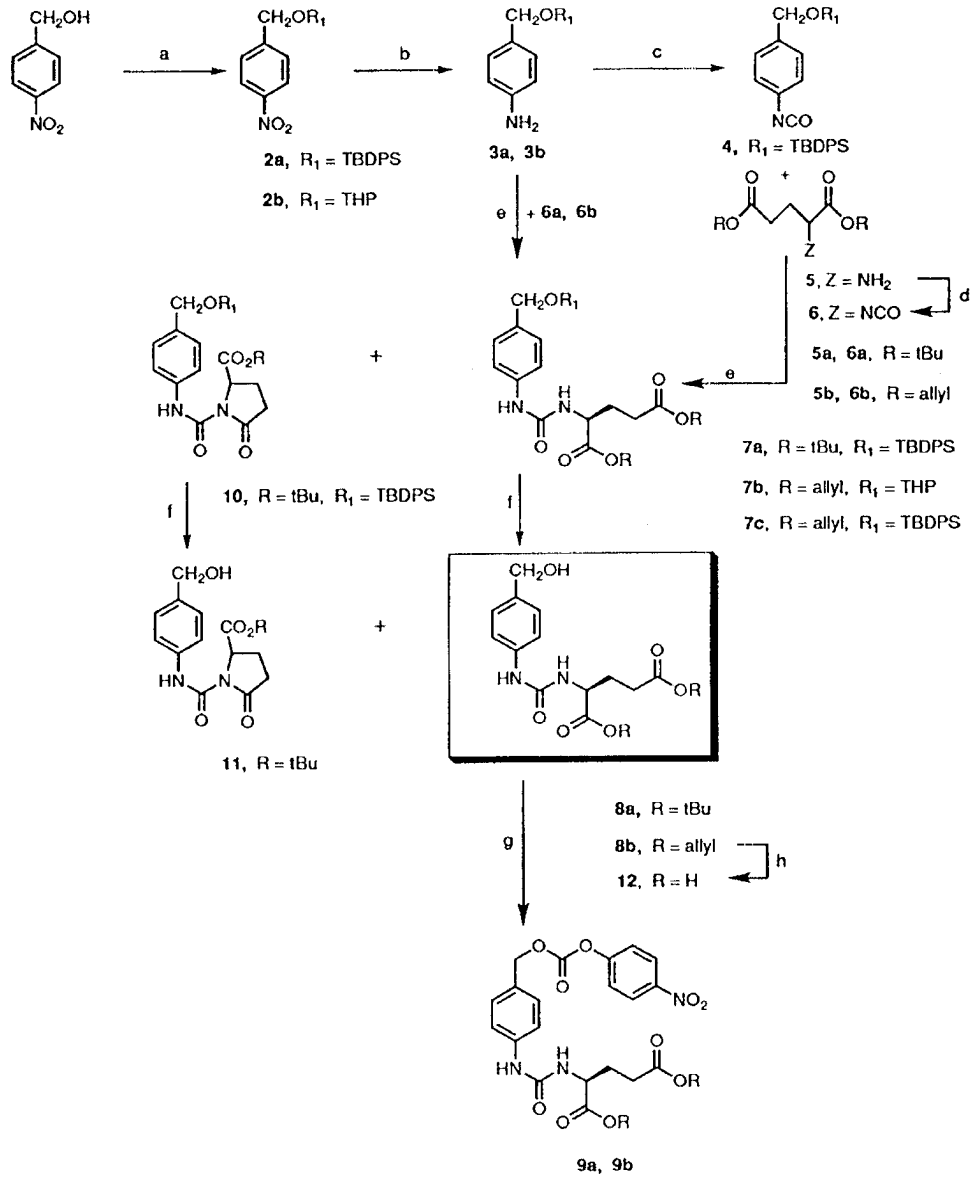
A broad panel of potential prodrugs with carbonate, carbamate, and ureido linkages were considered in order to find the optimal structural features responsible for the stability, substrate specificity for CPG2, and efficacy in cell lines transfected with the *stCPG2(Q)3* gene, as well as for further QSAR and optimization studies.

Chemistry

Two series of protected linkers (i.e., di-*tert*-butyl and diallyl L-glutamyl esters) were made since different deprotection strategies were needed to obtain the final prodrugs.

The starting material for **12**, 4-nitrobenzyl alcohol, was protected as *tert*-butyldiphenylsilyl ether **2a**[17,18] and reduced by hydrogen transfer (Pd/C (10%) and ammonium formate in EtOH) to the corresponding amine **3a**. The amine after conversion to isocyanate **4** (with triphosgene at 70 °C, in toluene) was coupled with di-*tert*-butyl L-glutamate, **5a**, in THF, at room temperature and in basic medium, leading to the protected linker **7a**. An alternative route to compound **7a** is the direct coupling of the amine **3a** with the di-*tert*-butyl L-glutamyl isocyanate **6a** under the conditions described above. Compound **6a** was obtained from di-*tert*-butyl L-glutamate, **5a**, by treatment with triphosgene and triethylamine at −78 °C in toluene. By exploiting this route, a one-pot procedure was devised to obtain compound **7a** directly, in good yield, starting from di-*tert*-butyl L-glutamate and amine **3a**. During this condensation the pyroglutamate analogue **10**, of compound **7a**, was formed as byproduct and was further deprotected with tetra-*n*-butylammonium fluoride in THF at room

Scheme 2[a]



[a] (a) TBDPSCl, imidazole, DMF (THF) or 3,4-dihydropyran, PPTS, CH_2Cl_2, rt; (b) Pd/C (10%), HCO_2NH_4, EtOH; (c) $(Cl_3CO)_2CO$, NEt_3, toluene, 70 °C; (d) $(Cl_3CO)_2CO$, NEt_3, toluene, −78 °C; (e) THF, NEt_3, rt; (f) Bu_4NF, THF, rt or AcOH, THF, H_2O; (g) 4-nitrophenyl chloroformate, CH_3CN or THF, NEt_3, rt; (h) $Pd(Ph_3P)_4$, pyrrolidine, rt.

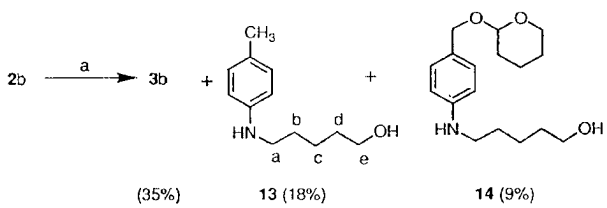
temperature to **11** (see Scheme 2). A similar deprotection afforded the linker **8a** which was purified by column chromatography and activated as 4-nitrophenyl carbonate **9a** (see Scheme 2).

The diallyl analogue **7c** was obtained using a different protection strategy, since the diallyl ester was unstable to tetra-*n*-butylammonium fluoride even at room temperature. The corresponding *O*-2′-tetrahydropyranyl ether **7b** was prepared by the sequence shown in Scheme 3. When the protected nitro derivative **2b** was reduced by hydrogen transfer to the amine **3b**, a mixture of compounds **3b**, **13**, and **14** resulted that were separated and purified by chromatography so that a yield of only 35−40% of the desired amine **3b** was obtained (see Scheme 3).

This amine was coupled with the diallyl L-glutamyl isocyanate **6b**, and the tetrahydropyranyl group was removed using AcOH in aqueous THF to give the linker **8a**, which was purified by column chromatography and activated as 4-nitrophenyl carbonate **9b** (see Scheme 2). The deprotected **12** was prepared from **8b** by the removal of the allylic groups with Pd(0) and pyrrolidine.

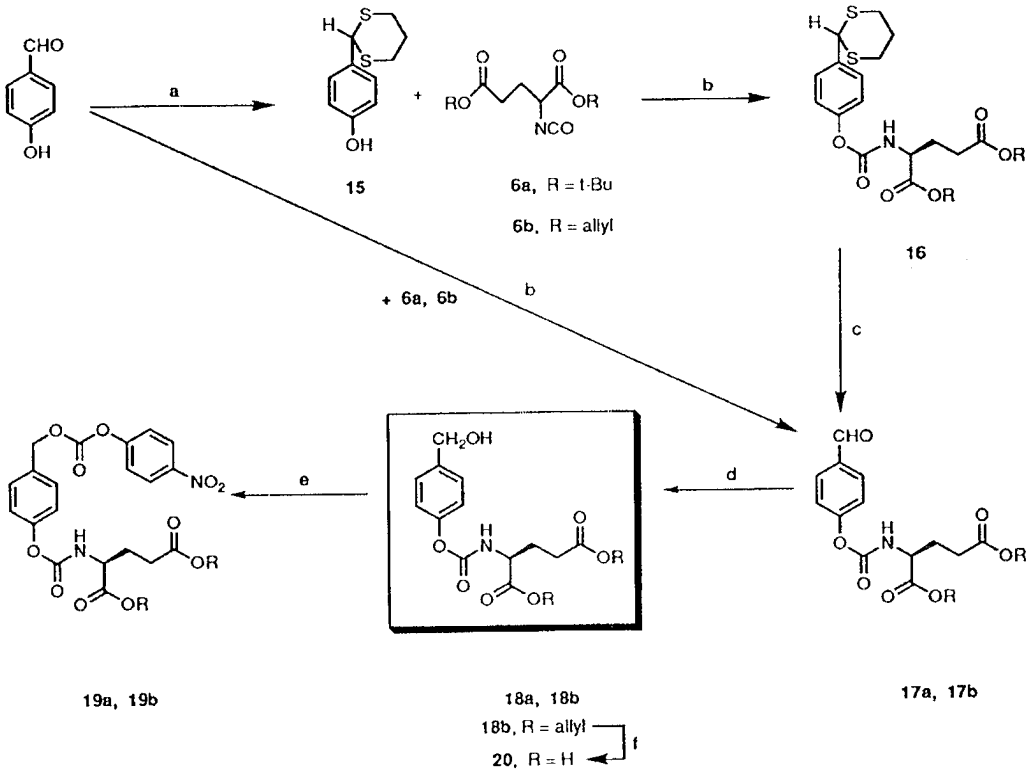
For the synthesis of the second self-immolative linker **20** (see Scheme 4), 4-hydroxybenzaldehyde was pro-

Scheme 3[a]



[a] (a) HCO_2NH_4, Pd/C, EtOH.

Scheme 4[a]



[a] (a) $HS(CH_2)_3SH$, $BF_3{\cdot}Et_2O$, $CHCl_3$, 20 °C; (b) NEt_3, toluene or $CHCl_3$, 20 °C; (c) $Hg(ClO_4)_2$, THF, 20 °C; (d) $NaBH_3CN$; (e) 4-nitrophenyl chloroformate, THF, NEt_3; (f) $Pd(Ph_3P)_4$, pyrrolidine, rt.

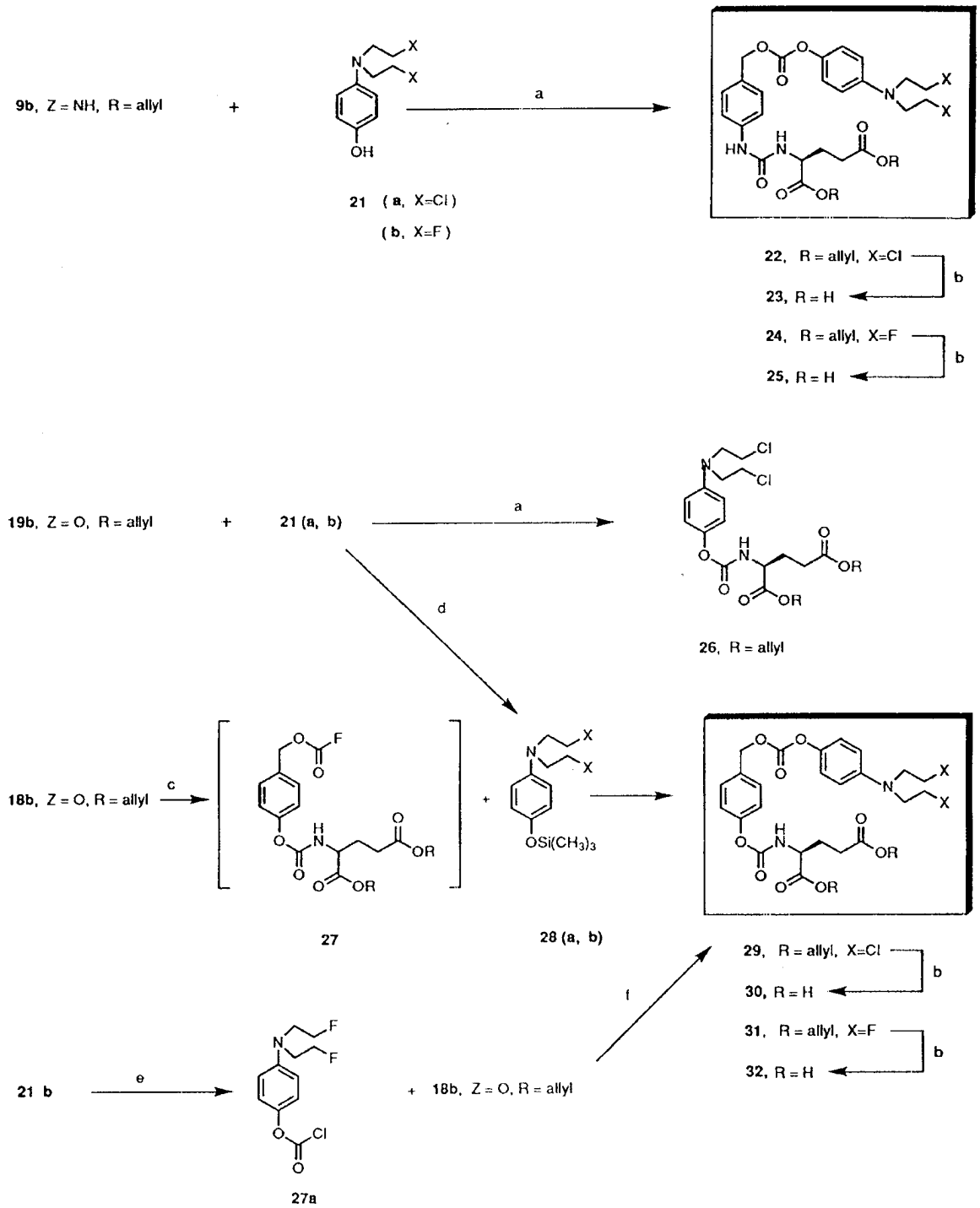
tected with 1,3-propanedithiol and boron trifluoride etherate, at room temperature, to give **15**[19] in very good yield. Reacting **15** with di-*tert*-butyl L-glutamyl isocyanate, **6a**, in toluene (NEt_3) and di-*tert*-butyl 4-[2′-(1′,3′-dithianyl)]phenoxycarbonyl-L-glutamate gave **16**. The deprotection of this intermediate with $Hg(ClO_4)_2$ in THF at room temperature led to the corresponding aldehyde **17a**. Subsequently, it was found that the aldehydes **17a,b** could be prepared by the direct coupling of the unprotected 4-hydroxybenzaldehyde with the isocyanates **6a,b**. The reduction of the aldehydes **17a,b** with sodium cyanoborohydride yielded the desired (4-hydroxymethylphenoxy)carbonyl-L-glutamates **18a,b**. Both linkers **18a,b** were activated as 4-nitrophenyl carbonates **19a,b**. The deprotection to **20** was achieved as described above for linker **12**.

The syntheses of aniline and phenol nitrogen mustard prodrugs **23**, **30**, **37**, and **40** and the corresponding fluorine mustard analogues **25**, **32**, **42**, and **44** require a new approach since the obvious route via the chloroformates of linkers **8a,b** and **18a,b** was not available. The failure of the direct phosgenation of some similar acylated benzylic derivatives has been reported previously.[20,21] Only one example of successful phosgenation of a similar 4-substituted benzylic alcohol has been claimed in a patent.[22] Our attempts to obtain chloroformates from the linkers using phosgene, diphosgene, or triphosgene under a variety of experimental conditions were also unsuccessful. The chloroformates are formed but are unstable above −40 °C[21] and difficult to handle. For this reason they were converted at −78 °C to the corresponding fluoroformates. Another difficulty lay with the deprotection procedures required to obtain the final prodrugs. The sensitivity of both carbamates and especially carbonates to basic and acidic media is widely recognized.[23]

The compounds fall into two categories: the phenol-derived and the aniline-derived self-immolative nitrogen mustards. Different procedures were employed to obtain the corresponding prodrugs **23**, **25**, **30**, and **32** from 4-bis(2-chloroethyl)- or 4-bis(2-fluoroethyl)aminophenol (**21a,b**) by condensation with the linkers **8a,b** and **18a,b**. Coupling **21a,b** with **9b** in the presence of the strongly basic anion exchanger Amberlyst 27 in acetonitrile resulted in the protected prodrugs **22** (X = Cl) and **24** (X = F). The removal of allylic groups, leading to the corresponding prodrugs **23** (X = Cl) and **25** (X = F), was achieved with Pd(0) and morpholine as allyl scavanger.

The same procedure failed when used with the activated linker **19b** leading unexpectedly to nitrogen mustard **26** (see Scheme 5). Therefore, a new procedure was designed taking advantage of the fluorine affinity for silicon.[24] Accordingly, linker **18b** was converted into the corresponding fluoroformate by reaction with phosgene, KF·HF, and 18-crown-6 ether at −78 °C in CH_2Cl_2 and coupled, at room temperature, in a one-pot procedure with the silylated nitrogen mustards **28a,b**. However, the coupling with the fluorine nitrogen mustard proceeded with very low yield (5%). Therefore the derivative **21b** was converted with phosgene to the corresponding chloroformate **27a** and coupled with linker **18b** to give compound **31** (X = F). The protected nitrogen mustard prodrugs **29** (X = Cl) and **31** (X = F) were deprotected to **30** (X = Cl) and **32** (X = F) with Pd(0) and morpholine (see Scheme 5).

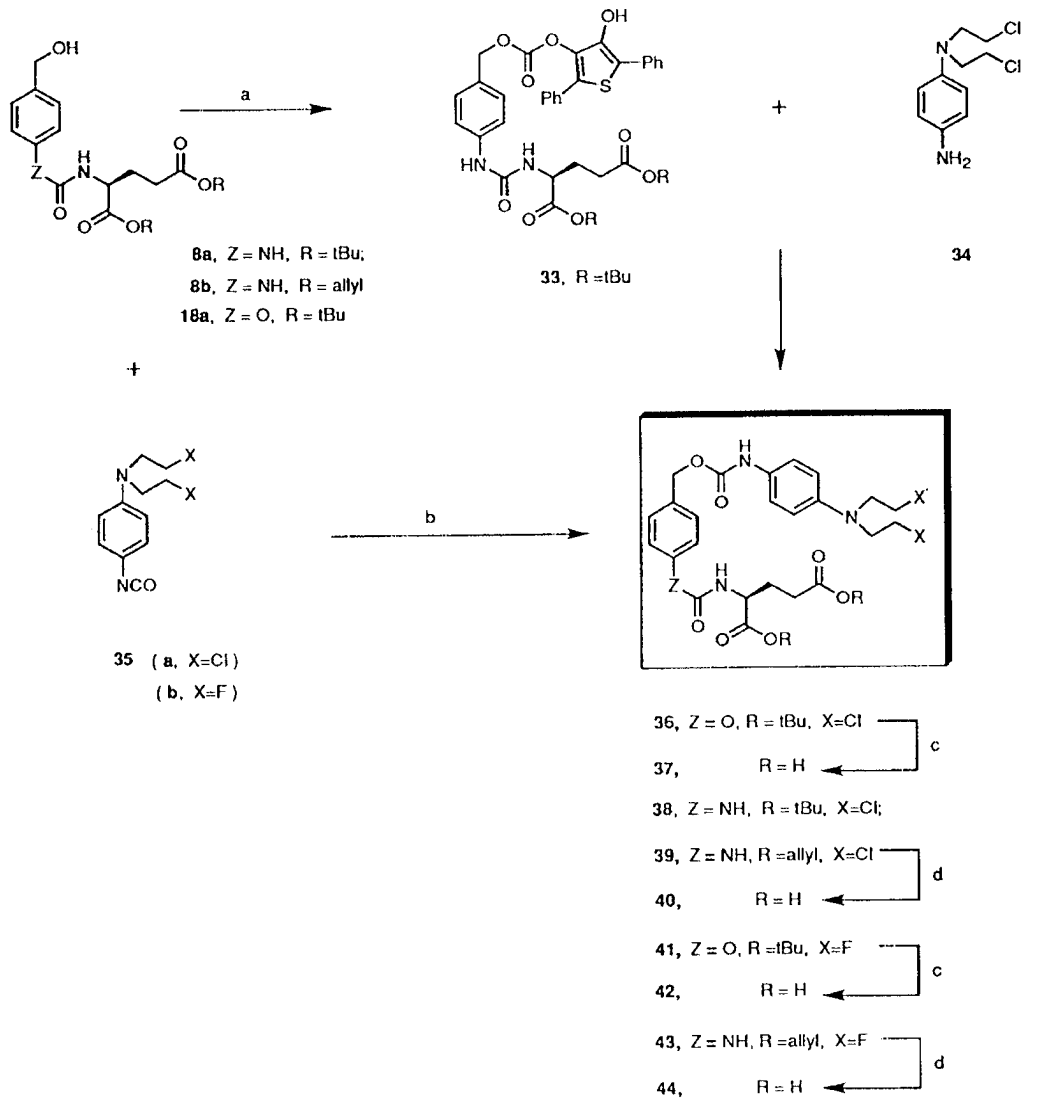
Scheme 5[a]



[a] (a) Amberlyst 27, CH_3CN; (b) $Pd(Ph_3)_4$, morpholine; (c) $COCl_2$, KF·HF, 18-crown-6, −78 °C; (d) Me_3SiCN; (e) $COCl_2$, NEt_3, CH_2Cl_2; (f) NEt_3, THF.

The aniline-derived self-immolative nitrogen mustards **37**, **40**, **42**, and **44** were obtained by a different route. In a first attempt the linker **8a** was activated by treatment with 4,6-diphenylthieno[3,4-*d*][1,3]dioxol-2-one 5,5-dioxide[25,26] to give the corresponding carbonate **33**. This intermediate is stable only at low temperature (−78 °C) and could not be isolated. However, the protected prodrug **38** was obtained in a one-pot procedure, using the nitrogen mustard **34**, in 10% yield (see Scheme 6). A more convenient procedure uses the 4-bis(2-chloroethyl)- or 4-bis(2-fluoroethyl)aminophenyl isocyanates (**35a,b**) (prepared from the corresponding aniline nitrogen mustards **34** (X = Cl) and **51** (X = F) with triphosgene) which were reacted with linkers **8a,b** and **18a** in the presence of dibutyltin dilaurate to give the protected prodrugs **36**, **39**, **41**, and **43**, respectively. The protected prodrugs **36** and **41** were deprotected to **37** and **42** with formic acid. The final deprotection of **38**

Scheme 6[a]



[a] (a) 4,6-Diphenylthieno[3,4-*d*][1,3]dioxol-2-one 5,5-dioxide; (b) dibutyltin dilaurate, toluene; (c) HCO_2H; (d) $Pd(Ph_3P)_4$, pyrrolidine.

with formic acid failed. Therefore, the self-immolative prodrugs **40** and **44** were obtained by deprotection with Pd(0) and pyrrolidine of the corresponding diallyl L-glutamates **39** and **43** (see Scheme 6).

The corresponding active drugs **21** (**a**, X = Cl; **b**, X = F), **34** (X = Cl), and **51** (X = F) were obtained according to Scheme 7. The starting materials were 4-fluoronitrobenzene for the aniline series and 4-amino-*O*-benzylphenol for the phenol series. The fluorinated nitrogen mustards were obtained by the nucleophilic displacement of the mesyl group (after mesylation of **46** and **48**) with KF and 18-crown-6 ether, for both series.

Physicochemical and Kinetic Data

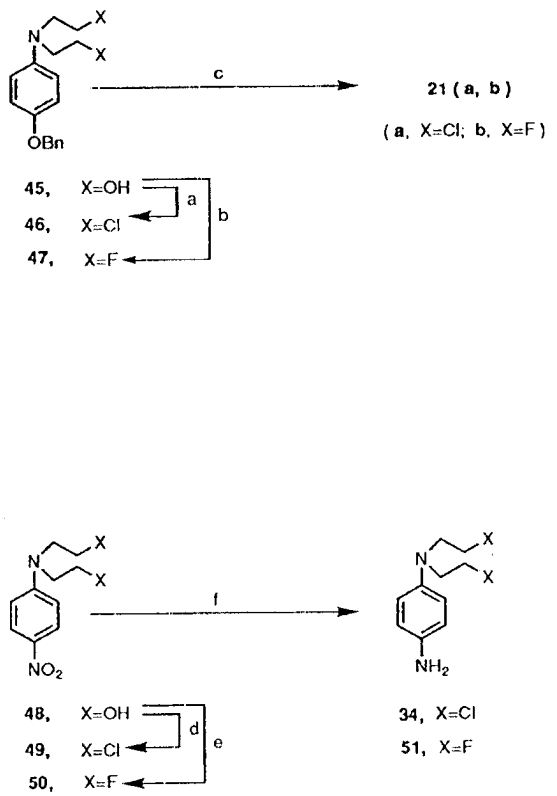
The chemical half-lives of the candidate prodrugs and parent drugs were determined by HPLC. The results are shown in Table 1. The K_M with CPG2 was determined for each of the novel potential prodrugs that had a $t_{1/2}$ greater than several minutes and for the deprotected linkers. The kinetic data are shown in Table 1.

The aqueous stability of the compounds (pH 7.4; 37 °C) varied considerably. The least stable prodrugs (**23**, **25**) showed little difference between the bischloro (**23**) and bisfluoro (**25**) congeners, implying that their instability lies elsewhere in the molecule. By contrast, and as expected, the bisfluoro compounds **42** and **44** were much more stable than their bischloro counterparts **37** and **40**. However, the relative stability of **30** as compared with **23** is surprising. It proved possible to determine the K_M's of the compounds **12**, **20**, **40**, **42**, and **44**. All were similar, in the <5 μM range. Prodrug **37** was shown to be a very poor substrate which required large amounts of enzyme (data not shown); thus full kinetic data could not be obtained. The k_{cat} for the isolated linker moieties **12** and **20** were determined.

Biological Evaluation

Cytotoxicity Assays. The synthesized prodrugs and some of the corresponding drugs were tested for cytotoxicity on the colon carcinoma cell line LS174T-stCPG2-(Q)3 made to express the surface-tethered CPG2. The plasmid pMCEF stCPG2(Q)3[15] encodes a CPG2 molecule that is expressed on the outer cell surface. It bears three asparagine-to-glutamine [(Q)3] mutations that

Scheme 7[a]



[a] (a) Mesyl chloride, Py, 70 °C; (b) mesyl anhydride, then KF, 18-crown-6, MeCN; (c) TFA, pentamethylbenzene; (d) $SOCl_2$, CH_2Cl_2; (e) mesyl anhydride, then F^-, $CHCl_3$; (f) H_2, Pd/C.

Table 1. Kinetic and Cytotoxicity (LS174T-stCPG2(Q)3) Data for Phenol and Aniline Self-Immolative Prodrugs and Their Bisfluoro Counterparts

compd	linker (L), prodrug (P), or drug (D)[a]	$t_{1/2}$, min	k_{cat},[b] s^{-1}	$t_{1/2}$, prodrug/drug	IC_{50}, μM
12	L1	602	+++[c]	NA	NA
20	L2	stable	+++[d]	NA	NA
23 (Cl)	P	2.9	+	0.3	0.48
25 (F)	P	2.4	+	NA	264.1
30 (Cl)	P	143	+	13.7	0.42
32 (F)	P	stable	+	NA	128.0
37 (Cl)	P	51	+	8.1	0.62 ± 0.21
40 (Cl)	P	48	++	7.6	0.46 ± 0.10
42 (F)	P	stable	++	NA	256.7
44 (F)	P	528	++	0.4	183.5
21a (Cl)	D	10.4	NA	NA	3.78 ± 1.83
34 (Cl)	D	6.3	NA	NA	0.34 ± 0.04
21b (F)	D	stable	NA	NA	322.9
51 (F)	D	1314	NA	NA	121.3
52 (Cl)	D	41	NA	NA	27.78 ± 1.27
CMDA	P	984	+++	17	24.74 ± 3.04

[a] This column describes the status of the compounds, being a prodrug (P), a drug (D), or a linker (L). [b] For +++, $k_{cat} > 50\ s^{-1}$; for ++, $k_{cat} = 50-10\ s^{-1}$; for +, $k_{cat} < 10\ s^{-1}$; for all, $K_M < 5\ \mu M$. [c] $k_{cat} = 65.4\ s^{-1}$ and $k_{cat}/K_M = 21.1\ s^{-1}\ \mu M$. [d] $k_{cat} = 140.0\ s^{-1}$ and $k_{cat}/K_M = 83.3\ s^{-1}\ \mu M$; NA, not applicable.

were otherwise inappropriately glycosylated. Two sublines were engineered and cloned. The control was transfected with the *LacZ* gene (LacZ), while the test line stCPG2(Q)3 expresses CPG2 tethered on the outer cell surface.[15] The LS174T-stCPG2(Q)3 cells were treated with the known prodrug CMDA for comparison with the synthesized prodrugs and the corresponding drugs. Expression of stCPG2(Q)3 in the LS174T results in an increased sensitivity to the treatment with CMDA compared with the control LS174T-LacZ-expressing cells. The IC_{50} of the assayed compounds against the stCPG2(Q)3-expressing cell clone, are presented in Table 1.

The phenol compounds (**25** and **30**) do not act as prodrugs, since they are as toxic as the corresponding drug in stCPG2(Q)3-expressing and LacZ-expressing cells. By contrast, the phenylenediamine compounds are successful prodrugs. Good IC_{50} prodrug/IC_{50} drug ratios of 20-fold (for **37**) and 33-fold (for **40**) and differentials of 12−14-fold between CPG2-expressing and control LacZ-expressing clones are achieved (see Figure 1A,B). The data that support these figures derive from the IC_{50} of the prodrug **37** in the control LS174T-LacZ line of 11.5 μM and the IC_{50} of the drug **34** in the test line of 0.34 μM resulting in a IC_{50} differential (11.5/0.34) of 33-fold. The result for the IC_{50} of the prodrug **40** in the control LS174T-LacZ line was 7 μM and the IC_{50} of the drug of 0.34 μM in the test line resulting in a IC_{50} differential (7/0.34) of 20-fold. The drugs released are between 7- and 70-fold more potent than the 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic acid (**52**) released by CMDA (see Figure 1C).

These data demonstrate the viability of this strategy and indicate that self-immolative prodrugs can be synthesized to release potent mustard drugs selectively by CPG2 tethered to the cell surface.

Summary

Four new potential self-immolative prodrugs derived from phenol and aniline nitrogen mustards, four model compounds derived from their corresponding fluorinated analogues, and two new self-immolative linkers, all substrates for CPG2, were designed and synthesized.

The phenylenediamine compounds were found to behave as prodrugs, yielding IC_{50} prodrug/IC_{50} drug ratios between 20- and 33-fold (for **37** and **40**) and differentials of 12−14-fold between CPG2-expressing and control LacZ-expressing clones. The drugs released are up to 70-fold more potent than 4-[(2-chloroethyl)-(2-mesyloxyethyl)amino]benzoic acid that results from the prodrug CMDA which has been used previously for GDEPT.

Experimental Section

All starting materials, reagents, and anhydrous solvents were purchased from Aldrich, unless otherwise stated. The di-*tert*-butyl L-glutamate was bought from Novabiochem and diallyl L-glutamate from Fluka. 4-Bis(2-hydroxyethyl)amino-nitrobenzene was obtained using the known procedure.[27]

Kieselgel 60 (0.043−0.060) was used in gravity columns (Art. 9385, Merck). TLC was performed on precoated sheets of Kieselgel 60 F254 (Art. 5735, Merck). Preparative HPLC was performed on an Axxial Chromatospac Prep 10 (Jobin-Yvon), using Merck Kieselgel 60 (0.015−0.040) (Art. 15,111). Reverse-phase HPLC was performed on a ThermoQuest system using a 5-mm C18300A column Jupiter Phenomenex. Melting points were determined on a Kofler hot-stage (Reichert Thermovar) melting point apparatus and are uncorrected. Low-resolution EI and FAB spectra were performed on a VG-2AB-SE double focusing magnetic sector mass spectrometer (Fisons Instruments, Warrington, U.K.), operating at a resolution of 1000. High-resolution accurate mass spectra were determined on the same system, but with a resolution set to

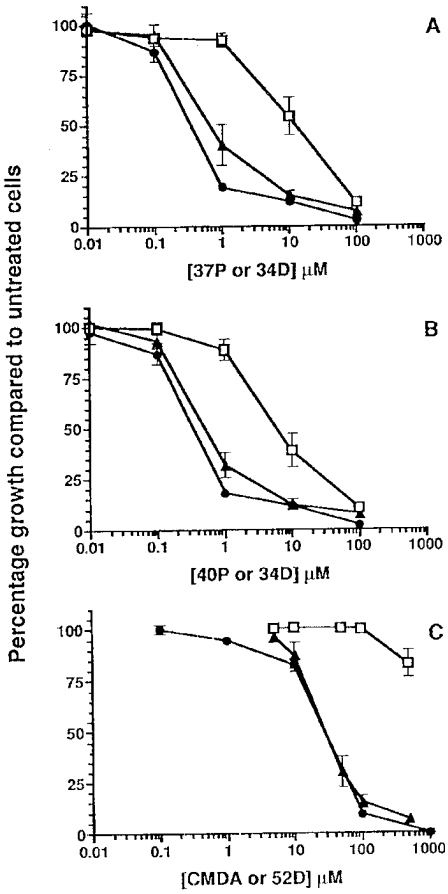


Figure 1. Sensitivity of LS174T cells expressing either control LacZ or stCPG2(Q)3 to challenge with prodrugs or drugs: (▲) cells expressing stCPG2(Q)3 in the presence of the prodrug; (●) cells expressing stCPG2(Q)3 in the presence of the drug; (□) cells expressing LacZ in the presence of the prodrug. Survival is determined by sulforhodamine assay, and the results are expressed as the proportion of cells surviving relative to similar untreated cells: A, incubation with prodrug **37** or drug **34** (mean of six separate experiments); B, incubation with prodrug **40** or drug **34** (mean of seven separate experiments); C, incubation with prodrug CMDA or drug **52** (mean of three separate experiments).

8000−10000. Masses are measured by peak matching the unknown with a mass of known composition. Reported spectra are by FAB unless otherwise stated. NMR spectra were determined in Me_2SO-d_6 on a 250-MHz spectrometer Brucker AC250 at 30 °C (303 K) unless otherwise stated. IR spectra (film) were recorded on a Perkin-Elmer 1720X FT-IR spectrometer. Elemental analyses were determined by Butterworth Laboratories Ltd. (Teddington, Middlesex, U.K.).

4-Nitrobenzyl *tert*-Butyldiphenylsilyl Ether (2a). To a stirred solution of 4-nitrobenzyl alcohol (1.00 g, 6.50 mmol) and imidazole (0.97 g, 14.10 mmol) in DMF (10.0 mL) was added *tert*-butyldiphenylchlorosilane (1.98 g, 1.87 mL, 7.20 mmol) over 10 min under nitrogen, at room temperature. The reaction mixture was stirred for an additional 5 h, diluted with 75 mL of Et_2O, washed with water (5 × 15 mL), dried ($MgSO_4$), and evaporated under vacuum. An oil, which crystallized on standing, was obtained. Recrystallization from aqueous 70% EtOH afforded 2.36 g (93%) of a white solid: mp 80−81 °C; ν_{max}/cm^{-1} (film) 2931, 2857 (CH_2), 1521, 1345 (NO_2); ^{1}H NMR δ_H 1.06 (s, 9H, t-Bu), 4.92 (s, 2H, $PhCH_2$), 7.42−7.46 (m, 5H, H_{arom}), 7.63−7.65 (m, 7H, Ph + $H_{arom2+6}$), 8.23 (d, 2H, $H_{arom3+5}$, J = 8.23 Hz); MS (EI) *m/z* 334 (M^+ − t-Bu, 100), 288 (M^+ − t-Bu − NO_2, 10), 256 (M^+ − t-Bu − Ph, 20), 199 (Ph_2SiOH^+, 100). Anal. ($C_{23}H_{25}NO_3Si$) C, H, N.

4-Nitrobenzyl 2′-Tetrahydropyranyl Ether (2b). To a stirred solution of 4-nitrobenzyl alcohol (1.53 g, 10.0 mmol) in CH_2Cl_2 (50 mL) were added 3,4-dihydropyran (1.36 mL, 1.26 g, 15.0 mmol) and pyridinium *p*-toluenesulfonate (0.25 g). After 1.5 h no starting material could be detected. The reaction mixture was evaporated to 4−5 mL, AcOEt (25 mL) added, and the solution washed with H_2O (3 × 50 mL) and brine, dried ($MgSO_4$), and evaporated under vacuum. The product **2b** (2.46 g, 99%) resulted as an oil: ^{1}H NMR δ_H 1.51−1.63 (m, 4H, $2H_{4'}$ + $2H_{5'}$), 1.66−1.74 (m, 2H, $2H_{3'}$), 3.50 (s, 1H, $H_{6'}$), 3.78 (m, 1H, $H_{6'}$), 4.62 (d, 1H, CH_2−Ph, J = 3.8 Hz), 4.72 (m, 1H, $H_{2'}$), 4.81 (d, 1H, CH_2−Ph), 7.62 (d, 2H, H_{arom}, J = 8.5 Hz), 8.22 (d, 2H, H_{arom}); MS *m/z* 260 (M^+ + 23, 15), 236 (M^+ − 1, 45), 136 (4-$NO_2C_6H_5CH_2^+$, 100).

4-Aminobenzyl *tert*-Butyldiphenylsilyl Ether (3a). To a stirred solution of **2a** (5.00 g, 12.80 mmol) in EtOH (100 mL) were added 10% Pd/C (1.50 g) and ammonium formate (4.60 g) in one portion at 20 °C. After 1.5 h no starting material could be detected. The catalyst was removed by filtration, the filtrate concentrated under vacuum, and the residue partitioned between EtOAc and H_2O. The organic layer was dried ($MgSO_4$) and concentrated to give **3a** (4.24 g, 91%) as an oil: ν_{max}/cm^{-1} (film) 3433, 3378 (NH_2), 2931, 2857 (CH_2); ^{1}H NMR δ_H 1.00 (s, 9H, t-Bu), 4.57 (s, 2H, $PhCH_2$), 4.98 (s broad, 2H, NH_2), 6.52 (d, 2H, $H_{arom3+5}$, J = 8.25 Hz), 6.96 (d, 2H, $H_{arom2+6}$), 7.42−7.46 (m, 5H, H_{arom}), 7.62−7.65 (m, 5H, H_{arom}); MS *m/z* 361 (M^+, 8), 304 (M^+ − t-Bu, 100), 199 (Ph_2SiOH^+, 100). Anal. ($C_{23}H_{27}NOSi$) C, H; N: calcd, 3.87; found, 4.33.

4-Aminobenzyl *O*-2′-Tetrahydropyranyl Ether (3b). The same procedure as for **3a**, starting from **2b** (8.53 g, 35.9 mmol), led to a three-component reaction mixture (6.40 g) after workup, which was separated by column chromatography (cyclohexane:AcOEt, 1:1). The desired compound **3b** eluted first as an oil (2.59 g, 38%): ν_{max}/cm^{-1} (film) 3447, 3360 (NH_2), 2943, 2870 (CH_2); ^{1}H NMR δ_H 1.42−1.53 (m, 4H, $2H_{4'}$ + $2H_{5'}$), 1.55−1.72 (m, 2H, $2H_{3'}$), 3.45 (s, 1H, $H_{6'}$), 3.79 (m, 1H, $H_{6'}$), 4.22 (d, 1H, $PhCH_2$, J = 11.1 Hz), 4.37 (d, 1H, $PhCH_2$), 4.60 (d, 1H, $H_{2'}$), 5.00 (s, 2H, NH_2), 6.52 (d, 2H, H_{arom}, J = 8.23 Hz), 6.97 (d, 2H, H_{arom}); MS (EI) *m/z* 207 (M^+, 100), 106 (H_2-$NC_6H_4CH_2^+$, 100). Anal. ($C_{12}H_{17}NO_2$) C, H, N.

The fraction eluting second, as an oil (1.15 g, 18%), was the **4-*N*-(5′-hydroxypentyl)toluidine, 13**: ν_{max}/cm^{-1} (film) 3353 (NH-, OH, broad), 2934, 2861 (CH_2); ^{1}H NMR δ_H* 1.39−1.55 (m, 6H, CH_{2b+c+d}), 2.13 (s, 3H, $PhCH_3$), 2.93 (q, 2H, CH_{2a}, J = 6.60 Hz), 3.39 (q, 2H, CH_{2e}, J = 6.10 Hz), 4.31 (t, 1H, OH or NH, J = 5.19 Hz), 5.19 (t, 1H, NH or OH), 6.45 (d, 2H, H_{arom}, J = 8.15 Hz), 6.86 (d, 2H, H_{arom}); MS (EI) *m/z* 193 (M^+, 18), 120 ($CH_3C_6H_4NHCH_2^+$, 100) (*for numbering see formula **13**).

The third fraction, eluting as an oil (0.88 g, 9%), was ***N*-(5′-hydroxypentyl)aminobenzyl 2′-*O*-tetrahydropyranyl ether, 14**: ν_{max}/cm^{-1} (film) 3367 (NH, OH, broad), 2937, 2863 (CH_2); ^{1}H NMR δ_H* 1.36−1.59 (m, 12H, $2H_b$ + $2H_c$ + $2H_d$ + $2H_{3'}$ + $2H_{4'}$ + $2H_{5'}$), 2.97 (q, 2H, $2H_a$, J = 5.91 Hz), 3.40 (q, 2H, $2H_e$), 3.50 (m, 1H, $H_{6'}$), 3.80 (m, 1H, $H_{6'}$), 4.24 (d, 1H, $PhCH_2$, J = 11.10 Hz), 4.32 (t, 1H, OH or NH, J = 5.23 Hz), 4.48 (d, 1H, $PhCH_2$), 4.60 (d, 1H, $H_{2'}$), 5.50 (t, 1H, NH or OH), 6.51 (d, 2H, H_{arom}, J = 8.81 Hz), 7.02 (d, 2H, H_{arom}); MS (EI) *m/z* 293 (M^+, 52), 192 ($C_5H_{10}NHC_6H_4CH_2^+$, 100) (*for numbering see formula **13**).

***tert*-Butyldiphenylsilyl 4-Oxymethylphenyl Isocyanate (4).** To a stirred solution of **3a** (0.63 g, 1.70 mmol) and triethylamine (0.25 mL, 0.18 g, 1.80 mmol) in toluene (10 mL) heated at 70 °C was added triphosgene (0.18 g, 0.61 mmol) in one portion. The reaction was monitored by IR (ν_{NCO} 2275 cm^{-1}). After 5 h the reaction mixture was filtered and the filtrate evaporated under vacuum, giving **4** (0.65 g, 99%) as an oil which was used without further purification: ν_{max}/cm^{-1} (film) 2931, 2857 (CH_2), 2275 (NCO); ^{1}H NMR δ_H 1.03 (s, 9H, t-Bu), 4.76 (s, 2H, $PhCH_2$), 7.23 (d, 2H, $H_{arom3+5}$, J = 8.38 Hz), 7.35 (d, 2H, $H_{arom2+6}$), 7.37−7.48 (m, 5H, H_{arom}), 7.62−7.71 (m, 5H, H_{arom}); MS (EI) *m/z* 330 (M^+ − t-Bu, 52), 286 (M^+ − t-Bu-NCO, 48), 199 (Ph_2SiOH^+, 100).

Di-*tert*-butyl *N*-[(4-{[*tert*-Butyldiphenylsilyl]oxymethyl}-phenyl)carbamoyl]-L-glutamate (7a) and *tert*-Butyl 1-

[*N*-(4-{[*tert*-butyldiphenylsilyl]oxymethyl}phenyl)-carbamoyl]-5-oxopyrrolidine-2-carboxylate (10). Method A: To a solution containing 6.03 g (13.10 mmol) of isocyanate **4** and 3.9 mL (2.64 g, 26.2 mmol) of triethylamine in 60 mL of THF was added at room temperature, during 30 min and under stirring, a solution of di-*tert*-butyl L-glutamate hydrochloride (3.90 g, 13.10 mmol) in 20 mL of THF. After 3 h the reaction was complete. The precipitate was filtered and the solvent removed under vacuum, leaving an oil. The oil was redissolved in AcOEt (25 mL), washed with H_2O (25 mL), aqueous HCl (2%) (25 mL), aqueous Na_2CO_3 (2%) (25 mL), and brine (2 × 25 mL), dried, and evaporated again giving 7.53 g of an oil. The product was purified by column chromatography (AcOEt:cyclohexane, 2:1). The first eluting compound was **7a** (5.31 g, 63%) as a solid: mp 89–90 °C (hexane); ν_{max}/cm^{-1} (film) 3359 (NH), 2932, 2857 (CH_2), 1729 (C=O, ester), 1670 (C=O, urea), 1154 (C–O); ^{1}H NMR δ_H 1.03 (s, 9H, t-Bu), 1.40 (s, 9H, t-Bu-G), 1.43 (s, 9H, t-Bu-G), 1.68–2.00 (2m, 2H, *CH*$_2$(NH)-CH), 2.18–2.32 (m, 2H, CH_2CO_2), 4.08–4.12 (m, 1H, *CH*(NH)-CH_2), 4.68 (s, 2H, $PhCH_2$), 6.38 (d, 1H, NH-G, J = 8.12 Hz), 7.19 (d, 2H, $H_{arom3+5}$, J = 8.41 Hz), 7.32–7.47 (m, 7H, H_{arom} + $H_{arom2+6}$), 7.62–7.70 (m, 5H, H_{arom}), 8.54 (s, 1H, NH–Ph); MS (EI) *m*/*z* 590 (M^+ – t-Bu + 1, 2), 534 (M^+ – 2t-Bu, 5), 478 (M – 3t-Bu, 100), 199 (Ph_2SiOH^+, 100). Anal. ($C_{37}H_{50}N_2O_6Si$) C, H, N.

The compound eluting second was **10**, an oil (0.39 g, 5%), which was deprotected without further characterization.

Method B (one-pot synthesis of compound 7a): To a solution of di-*tert*-butyl L-glutamate hydrochloride (4.14 g, 14.0 mmol) and triphosgene (1.39 g, 4.67 mmol) in toluene, cooled at −78 °C, was added triethylamine (3.90 mL, 2.83 g, 28 mmol) in toluene (10 mL) dropwise over 30 min. The reaction ws allowed to reach room temperature and finished in 50 min (monitored by IR, ν_{NCO} 2253 cm^{-1}). To this mixture was added a solution containing 4-aminobenzyl *tert*-butyldiphenylsilyl ether (**3a**) (5.00 g, 13.8 mmol) and triethylamine (1.95 mL, 14.0 mmol) in 30 mL of toluene over 5–10 min. The reaction was monitored by IR (disappearance of the ν_{NCO} 2253 cm^{-1} peak) and was finished in 14–20 h. The reaction mixture was filtered, washed with H_2O (200 mL), aqueous HCl (1%) (200 mL), aqueous Na_2CO_3 (1%) (200 mL), and H_2O (2 × 200 mL), dried ($MgSO_4$), and evaporated under vacuum to give an oil (9.90 g). The product was deprotected without further purification.

Diprop-2-enyl *N*-[(4-{[2′-*O*-tetrahydropyranyl]oxymethyl}phenyl)carbamoyl]-L-glutamate (7b) was obtained, starting from diallyl L-glutamate *p*-toluenesulfonate (1.08 g, 3.1 mmol) and **3b** (0.52 g, 2.5 mmol) by the one-pot procedure. An oil resulted which was separated by preparative HPLC using cyclohexane:AcOEt (1.5:1) as eluent (0.75 g, 65%): ν_{max}/cm^{-1} (film) 3362 (NH_2), 2944, 2871 (CH_2); ^{1}H NMR δ_H 1.47–1.50 (m, 4H, $2H_{4'}$ + $2H_{5'}$), 1.60–1.88 (m, 3H, $2H_{3'}$ + *CH*$_2$(NH)-CH), 1.88–2.05 (m, 1H, –*CH*$_2$CH(NH)–), 2.37–2.45 (m, 2H, CH_2CO_2), 3.45 (s, 1H, $H_{6'}$), 3.80 (m, 1H, $H_{6'}$), 4.20–4.31 (m, 1H, *CH*(NH)CH_2), 4.35 (d, 1H, $PhCH_2$, J = 11.55 Hz), 4.53–4.63 (m, 5H, $PhCH_2$ + CH_2O, allyl), 5.18–5.33 (m, 4H, CH_2= allyl), 5.80–6.00 (m, 2H, CH= allyl), 6.60 (d, 1H, NH-G, J = 8.03 Hz,), 7.20 (d, 2H, H_{arom}, J = 8.47 Hz), 7.35 (d, 2H, H_{arom}), 8.47 (s, 1H, NH–Ph); MS (EI) *m*/*z* 461 (M^+ + 1, 15). Mass ($C_{24}H_{32}N_2O_7Na$) calcd, 483.2107; found, 483.2120.

Diprop-2-enyl *N*-[(4-{[*tert*-butyldiphenylsilyl]oxymethyl}phenyl)carbamoyl]-L-glutamate (7c) was synthesized by the same method starting from 5.0 g (13.8 mmol) of amine **3a**. An oil resulted which was purified by column chromatography (cyclohexane:AcOEt, 2:1) leading to **7c** (6.47 g, 47%): ν_{max}/cm^{-1} (film) 3382 (NH_2), 1739 (C=O, ester), 1651 (C=O, amide); ^{1}H NMR δ_H 1.03 (s, 9H, t-Bu), 1.85–2.11 (2m, 2H, *CH*$_2$(NH)CH), 2.46–2.52 (m, 2H, CH_2CO_2), 4.32–4.35 (m, 1H, *CH*(NH)CH_2), 4.56 (dd, 2H, CH_2= allyl, J = 4.62 Hz), 4.62 (dd, 2H, CH_2= allyl), 4.69 (s, 2H, $PhCH_2$), 5.17–5.38 (m, 4H, CH_2O allyl), 5.80–6.00 (m, 2H, CH= allyl), 6.60 (d, 1H, NH-G, J = 8.03 Hz), 7.20 (d, 2H, $H_{arom3+5}$, J = 8.49 Hz), 7.34–7.48 (m, 7H, H_{arom} + $H_{arom2+6}$), 7.63–7.72 (m, 5H, H_{arom}), 8.57 (s, 1H, NH–Ph).

Di-*tert*-butyl *N*-[(4-{Hydroxymethyl}phenyl)carbamoyl]-L-glutamate (8a). Compound **7a** (5.15 g, 8.0 mmol) was dissolved in THF (100 mL) and tetrabutylammonium fluoride solution in THF (1 M, 20.0 mL, 2.5 equiv) was added, in one portion, under stirring at room temperature. The reaction was finished in 3 h. The reaction mixture was evaporated under vacuum. The residue was dissolved in AcOEt (50 mL), washed with H_2O (3 × 100 mL), dried ($MgSO_4$), and evaporated again. A yellow oil (5.08 g) resulted which was purified by column chromatography (AcOEt:cyclohexane, 3:1) yielding an oil (1.88 g, 58%) which crystallized on standing: mp 103–4 °C (aqueous MeOH, 60%); ν_{max}/cm^{-1} (film) 3370 (broad, NH+OH), 2967 (CH_3), 2930, 2857 (CH_2), 1716 (C=O, ester), 1678 (C=O, amide), 1153 (C–O). Anal. ($C_{21}H_{32}N_2O_6$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

The fraction eluting second was ***tert*-butyl 1-[*N*-(4-{hydroxymethyl}phenyl)carbamoyl]-5-oxopyrrolidine-2-carboxylate (11)** (0.356 g, 13%): white solid, mp 157–9 °C; ν_{max}/cm^{-1} (film) 3354 (NH_2), 2977, 2932 (CH_2), 1719 (C=O, ester), 1676 (C=O, amide); ^{1}H NMR δ_H 1.41 (s, 9H, t-Bu), 1.90–2.08 (2m, 2H, *CH*$_2$(NH)CH), 2.37 (t, 2H, CH_2CO, J = 7.67 Hz), 4.23 (t, 1H, *CH*(N)CH_2), 4.52 (d, 2H, CH_2, J = 5.64 Hz), 5.23 (t, 1H, CH_2OH), 7.28 (d, 2H, $H_{arom3+5}$, J = 8.36 Hz), 7.39 (d, 2H, $H_{arom2+6}$), 8.44 (s, 1H, PhNH); MS (EI) *m*/*z* 334 (M^+, 16), 278 (M^+ – t-Bu, 82). Anal. ($C_{17}H_{22}N_2O_5$) C, H; N: calcd, 8.38; found, 7.92.

Diprop-2-enyl *N*-[(4-{Hydroxymethyl}phenyl)carbamoyl]-L-glutamate (8b). The intermediate **7b** (0.610 g, 1.3 mmol) was hydrolyzed at 45 °C in a mixture of AcOH:THF:H_2O (24.5 mL, 4:2:1) for 3.5 h. The reaction mixture was diluted with H_2O (50 mL) and extracted with ether (2 × 25 mL) and then with AcOEt (2 × 30 mL). The pooled organic layers were washed with H_2O (2 × 30 mL), dried ($MgSO_4$), and evaporated to dryness (with addition of toluene, 2 × 30 mL). An oil resulted which was purified by preparative HPLC (cyclohexane:AcOEt, 1:2), leading to **8b** (0.221 g, 45%): ν_{max}/cm^{-1} (film) 3354 (NH_2, OH, broad), 1737 (C=O, ester), 1659 (C=O, amide). Anal. ($C_{19}H_{24}N_2O_6$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[4-Nitrophenoxycarbonyloxy]-methyl}phenyl)carbamoyl]-L-glutamate (9a). To a stirred solution of **8a** (0.200 g, 0.49 mmol) in dry THF (10 mL) were added 4-nitrophenyl chloroformate (0.11 g, 0.5 mmol) and triethylamine (0.1 mL, 0.6 mmol) at room temperature. The reaction was complete after 1 h. The formed precipitate was filtered and the solution concentrated under vacuum. AcOEt (10 mL) was added; the solution was washed with brine (2 × 10 mL), dried ($MgSO_4$), and evaporated again, giving an oil which was purified by column chromatography (0.160 g, 57%) and repurified by preparative HPLC (0.140 g, 50%): mp 55–6 °C; ν_{max}/cm^{-1} (film) 3349 (NH_2), 2979, 2932 (CH_2), 1767 (C=O, carbonate), 1716 (C=O, ester), 1652 (C=O, amide), 1527, 1349 (NO_2). Mass ($C_{28}H_{36}N_3O_{10}$) calcd, 574.2401; found, 574.2420. Anal. ($C_{28}H_{35}N_3O_{10}$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-Nitrophenoxycarbonyloxy]-methyl}phenyl)carbamoyl]-L-glutamate (9b). Starting from **8b** (0.190 g, 0.50 mmol), **9b**, obtained by the same procedure as **9a**, was purified by preparative HPLC to a solid (0.132 g, 48.6%): mp 106–7 °C; ν_{max}/cm^{-1} (film) 3356 (NH_2), 2933 (CH_2), 1766 (C=O, carbonate), 1738 (C=O, ester), 1660 (C=O, amide), 1525, 1346 (NO_2). Mass ($C_{26}H_{27}N_3O_{10}Na$) calcd, 564.1594; found, 564.1590. Anal. ($C_{26}H_{27}N_3O_{10}$) H, N; C: calcd, 57.67; found, 58.09. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

[(4-Hydroxymethylphenyl)carbamoyl]-L-glutamic Acid (12). In the stirred solution of **8b** (0.200 g, 0.53 mmol) in CH_2Cl_2 (15 mL) were added $Pd(PPh_3)_4$ (40 mg) and pyrrolidine (1 mL, 11.8 mmol). On addition of pyrrolidine, a precipitate started to form immediately. After 40 min of stirring, the solvent was removed and the precipitate washed with AcOEt and CH_2Cl_2. The residue was dissolved in methanol (10 mL) and passed through a weakly acid resin IRC50 ion-exchange column. After eluting with methanol (50 mL), the eluate was evaporated under vacuum to yield **12** (0.135 g, 86%) as a solid;

mp 73–6 °C. Mass ($C_{13}H_{17}N_2O_6$) calcd, 297.1087; found, 297.1070. Anal. ($C_{13}H_{16}N_2O_6$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{1,3-Dithian-2-yl}phenyloxy)carbonyl]-L-glutamate (16). Compound **16** was prepared by the one-pot procedure (as described above for compound **7a**, method B) from di-*tert*-butyl L-glutamate hydrochloride (1.50 g, 5.0 mmol) and 4-[2′-(1′,3′-dithianyl)]phenol, **15** (1.10 g, 5.0 mmol). The reaction was left for 12 h. The solid formed was filtered; the solution was washed with aqueous NaOH (1%) (75 mL) and H_2O (2 × 75 mL), dried ($MgSO_4$), and evaporated under vacuum to yield a solid (2.30 g, 95.8%). Recrystallization from aqueous 50% EtOH afforded **16** (1.81 g, 72.7%): mp 137–8 °C; ν_{max}/cm^{-1} (film) 3347 (NH_2), 2977, 2945 (CH_2), 1728 (C=O, ester); ^{1}H NMR δ_H 1.41 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.62–1.90 (m, 2H, $2H_{5'}$), 1.90–2.32 (2m, 2H, CH_2(NH)CH), 2.30–2.40 (m, 2H, CH_2CO_2), 2.86–2.93 (m, 2H, $2H_{4'or6'}$), 3.09 (t, 2H, $2H_{6'or4'}$, J = 12.3 Hz), 5.41 (s, 1H, $H_{2'}$), 7.08 (d, 2H, $H_{arom3+5}$, J = 8.50 Hz), 7.43 (d, 2H, $H_{arom2+6}$), 8.12 (d, 1H, NH-G, J = 7.81 Hz); MS (EI) *m/z* 497 (M^+, 2), 451 (M^+ – t-Bu, 2), 359 (M^+ – 2t-Bu, 20). Anal. ($C_{24}H_{25}NO_6S_2$) C, N; H: calcd, 7.09; found, 6.68.

Di-*tert*-butyl *N*-[(4-Formylphenyloxy)carbonyl]-L-glutamate (17a). Procedure A: To a stirred solution of dithiane **16** (0.50 g, 1.0 mmol) in $CHCl_3$ (10 mL) and THF (5 mL) was added dropwise a solution of $Hg(ClO_4)_2$·$3H_2O$ (0.91 g, 2.0 mmol) in THF (5 mL). The reaction was completed in 5 min. The precipitate was filtered and the solution washed with H_2O (2 × 25 mL), aqueous Na_2CO_3 (25 mL), and H_2O (2 × 25 mL), dried ($MgSO_4$), and evaporated under vacuum. The compound **17a** (0.33 g, 81%) was obtained as a clear oil.

Procedure B: To a solution of di-*tert*-butyl L-glutamyl isocyanate **6a** (prepared from di-*tert*-butyl L-glutamate hydrochloride (0.200 g, 0.67 mmol), triphosgene (0.067 g, 0.22 mmol), and triethylamine (0.190 mL, 1.35 mmol) according to the procedure described above) in $CHCl_3$ (15 mL) were added at room temperature 4-hydroxybenzaldehyde (0.082 g, 0.67 mmol) and triethylamine (0.190 mL, 1.35 mmol) in $CHCl_3$ (10 mL) over 10 min. The reaction was heated at reflux for 2 h, when the ν_{NCO} peak disappeared. The solvent was removed under vacuum and the compound purified by preparative HPLC (cyclohexane:AcOEt, 6:1). A clear oil resulted (0.197 g, 72%): ν_{max}/cm^{-1} (film) 3347 (NH_2), 2979, 2934 (CH_2), 1731 (C=O, ester), 1712 (C=O, aldehyde); ^{1}H NMR δ_H 1.41 (s, 9H, t-Bu), 1.43 (s, 9H, t-Bu), 1.80–2.02 (2m, 2H, CH_2(NH)CH), 2.32–2.39 (m, 2H, CH_2CO_2), 3.99–4.04 (m, 1H, –CH(NH)CH_2–), 7.34 (d, 2H, $H_{arom3+5}$, J = 8.39 Hz), 7.96 (d, 2H, $H_{arom2+6}$), 8.29 (d, 1H, NH-G, J = 7.72 Hz), 9.98 (s, 1H, CHO). Anal. ($C_{21}H_{29}NO_7$) C, H, N.

Diprop-2-enyl *N*-[(4-formylphenyloxy)carbonyl]-L-glutamate (17b) was obtained as a clear oil (2.90 g, 76%) using procedure B: ν_{max}/cm^{-1} (film) 3347 (NH_2), 2979, 2934 (CH_2), 1731 (C=O, ester), 1712 (C=O, aldehyde); ^{1}H NMR δ_H 1.93–2.16 (2m, 2H, CH_2(NH)CH), 2.53 (t, 2H, CH_2CO_2), 4.19–4.26 (m, 1H, CH(NH)CH_2), 4.57 (d, 2H, CH_2-allyl, J = 5.35 Hz), 4.64 (d, 2H, CH_2-allyl), 5.18–5.37 (m, 4H, CH_2=allyl, J = 5.35 Hz), 5.86–5.94 (m, 2H, CH=allyl), 7.35 (d, 2H, $H_{arom3+5}$, J = 8.50 Hz), 7.77 (d, 2H, $H_{arom2+6}$), 8.49 (d, 1H, NH-G, J = 7.77 Hz), 9.98 (s, 1H, CHO); MS *m/z* 398 (M^+ + 23, 16), 376 (M^+ + 1, 14). Anal. ($C_{19}H_{21}NO_7$) C, H; N: calcd, 3.73; found, 4.16.

Di-*tert*-butyl *N*-[(4-{Hydroxymethyl}phenoxy)carbonyl]-L-glutamate (18a). Compound **17a** (0.150 g, 0.36 mmol) dissolved in a mixture of H_2O:AcOH:CH_3OH (5.2 mL, 1:1:1) was reduced with sodium cyanoborohydride (0.027 g, 0.43 mmol) at room temperature. The reaction was complete after 1 h. AcOEt (20 mL) was added, the reaction mixture evaporated under vacuum, the residue dissolved in AcOEt (20 mL), and the solution washed with H_2O (20 mL) and brine (2 × 20 mL), dried ($MgSO_4$), and evaporated to dryness. Compound **18a** (0.127 g, 86%) was obtained as an oil. The final purification was achieved by preparative HPLC (cyclohexane:AcOEt, 2:1): ν_{max}/cm^{-1} (film) 3352 (NH_2, OH, broad), 2979, 2945 (CH_2), 1728 (C=O, ester), 1712 (C=O, aldehyde). Mass ($C_{21}H_{31}NO_7$-Na) calcd, 432.1998; found, 432.1990. Anal. ($C_{21}H_{31}NO_7$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{Hydroxymethyl}phenoxy)carbonyl]-L-glutamate (18b). Compound **18b** was obtained by a similar reduction procedure as a clear oil (0.233 g, 78%). Mass ($C_{19}H_{23}NO_7Na$) calcd, 400.1372; found, 400.1376. Anal. ($C_{28}H_{35}N_3O_{10}$) H, N; C: calcd, 60.47; found, 60.01. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[4-Nitrophenoxycarbonyloxy]-methyl}phenoxy)carbonyl]-L-glutamate (19a). Activation of linker **18a** (1.0 g, 2.7 mmol) was achieved by the same procedure as for **9a**. After purification, an oil resulted (0.64 g, 41%): ν_{max}/cm^{-1} (film) 3367 (NH_2), 2980, 2933 (CH_2), 1767 (C=O, carbonate), 1728 (C=O, ester). Anal. ($C_{28}H_{34}N_2O_{11}$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Starting from **18b** (0.4 g, 1.06 mmol), **19b** was obtained by the same method as an oil (0.448 g, 75.5%). Mass ($C_{26}H_{26}N_2O_{11}$-Na) calcd, 565.1434; found, 565.1430. Anal. ($C_{26}H_{26}N_2O_{11}$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

[(4-Hydroxymethylphenyl)oxycarbonyl]-L-glutamic Acid (20). Starting from **18b** (150 mg, 0.4 mmol) in CH_2Cl_2 (12 mL) and using $Pd(PPh_3)_4$ (25 mg) and pyrrolidine (152 mL, 1.8 mmol), compound **20** was obtained by the procedure described above for compound **12**. However, the compound eluting from the IRC50 ion-exchange column, after evaporation under vacuum, led only to an impure **20** (103 mg). This product was purified by reverse-phase (C18) preparative HPLC using as mobile phase a gradient of 0.1% aqueous TFA/acetonitrile. After lyophilization **20** (36 mg, 30%) was obtained as a white powder: mp 135–7 °C. Mass ($C_{13}H_{15}NO_7Na$) calcd, 320.0746; found, 320.0730. Anal. ($C_{13}H_{15}NO_7$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-chloroethyl}amino)-phenoxy carbonyloxy]methyl}phenyl)carbamoyl]-L-glutamate (22). The solution containing the activated linker **9b** (0.200 g, 0.37 mmol), 4-bis(2-chloroethyl)aminophenol (**21a** (X = Cl)), (0.140 g, 0.60 mmol) dissolved in CH_3CN (10 mL), and a strongly basic resin-Amberlyst 27 HO^- (0.13 g) was stirred for 20 h. The resin was filtered off, and a further 0.080 g of fresh resin was added. After 30 min of stirring, the second portion of resin was filtered off and the solvent evaporated. The product was purified by column chromatography (cyclohexane:AcOEt, 2:1) to afford **28** (82 mg, 32%) as a solid: mp 134–5 °C. Mass ($C_{30}H_{35}N_3O_8Cl_2Na$) calcd, 658.1699; found, 658.1690. Anal. ($C_{30}H_{35}N_3O_8Cl_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-fluoroethyl}amino)-phenoxycarbonyloxy]methyl}phenyl)carbamoyl]-L-glutamate (24). 24 (120 mg, 40%) was obtained as a solid, mp 129–31 °C, by a similar procedure as **22**. Mass ($C_{30}H_{36}N_3O_8F_2$) calcd, 604.2470; found, 604.2450. Anal. ($C_{30}H_{35}N_3O_8F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenyl)carbamoyl]-L-glutamic Acid (23).** To a solution of **22** (0.200 g, 0.32 mmol) and $Pd(PPh_3)_4$ (25 mg, 0.022 mmol) in CH_2Cl_2 (9 mL) was added morpholine (107 mL, 1.23 mmol), and the mixture stirred for 2 h under argon at room temperature. The reaction mixture was diluted with AcOEt (a precipitate formed upon addition of AcOEt). The solvent was evaporated, and the insoluble residue was washed with AcOEt. It was dissolved in methanol (10 mL) and passed through a weakly acid resin, IRC50 ion-exchange column. After eluting with methanol (50 mL), the eluate was evaporated under vacuum to yield **23** (0.145 g, 81%) as a solid: mp 73–6 °C. Mass ($C_{24}H_{27}N_3O_8Cl_2Na$) calcd, 578.1073; found, 578.1070. Anal. ($C_{24}H_{27}N_3O_8Cl_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-fluoroethyl}amino)phenoxycarbonyl-oxy]methyl}phenyl)carbamoyl]-L-glutamic acid (25)** was

obtained as a white solid (68 mg, 72%), mp 104–7 °C, by a procedure similar to **23**. Mass ($C_{24}H_{27}N_3O_8F_2Na$) calcd, 546.1664; found, 546.1680. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

4-[Bis(2-fluoroethyl)amino]phenyl Chloroformate (27a). Over a solution of **21b** (0.2 g, 1.0 mmol) in CH_2Cl_2 (8 mL) phosgene (20% in toluene, 3.5 mL, 7 mmol) was added. After 5 min of stirring, a solution of NEt_3 (0.14 mL, 1 mmol) in CH_2Cl_2 (8 mL) was added dropwise over 15 min. The solvent was evaporated, the residue taken up in AcOEt, the precipitate filtered off, and the filtrate evaporate to dryness to afford **27a** (0.184 g, 74.5%) as an oil: IR ν_{max}/cm^{-1} (film) 1780 (OCOCl), 1515, 1117; ^{1}H NMR ($CDCl_3$) δ_H 3.73 (2t, 4H, NCH_2, J = 5.20 Hz, J_{H-F} = 23.63 Hz), 4.61 (2t, 4H, CH_2F, J = 5.22 Hz, J_{H-F} = 47.15 Hz), 6.68 (d, 2H, $H_{arom2+6}$, J = 9.26 Hz), 7.08 (d, 2H, $H_{arom3+5}$).

4-Bis(2-chloroethyl)aminophenyl Trimethylsilyl Ether (28a). Phenol nitrogen mustard **21a** (X = Cl) (60 mg, 0.26 mmol) and trimethylsilyl cyanide (40 mL, 0.3 mmol) were stirred at room temperature without solvent for 30 min. Boiling hexane (10 mL) was added to the reaction mixture and the remaining solid filtered off. After evaporation, **28a** (70 mg, 88%) as an oil resulted: ^{1}H NMR δ_H 0.20 (s, 9H, $Si(CH_3)_3$), 3.55–3.70 (m, 8H, $N(CH_2CH_2Cl)_2$), 6.65 (d, 2H, J = 9.30 Hz, $H_{arom2+6}$), 6.73 (d, 2H, $H_{arom3+5}$); MS *m/z* 305 (M^+, 100), 270 (M^+ – Cl, 35). Mass ($C_{13}H_{21}NOCl_2Si$) calcd, 305.0769; found, 305.0765. Anal. ($C_{13}H_{21}NOCl_2Si$) C, H, N, Cl.

4-Bis(2-fluoroethyl)aminophenyl Trimethylsilyl Ether (28b). Method A: 28b (0.65 g, 80%) was synthesized according to the same procedure as **21b** but using excess trimethylsilyl cyanide and longer time periods: ^{1}H NMR δ_H 0.19 (s, 9H, $Si(CH_3)_3$), 3.61 (2t, 4H, NCH_2, J = 5.20 Hz, J_{H-F} = 24.44 Hz), 4.54 (2t, 4H, CH_2F, J = 5.17 Hz, J_{H-F} = 47.55 Hz), 6.68 (s, 4H, H_{arom}); MS *m/z* 273 (M^+, 100), 254 (M^+ – F, 23). Mass ($C_{13}H_{21}NOF_2Si$) calcd, 273.1360; found, 273.1345. Anal. ($C_{13}H_{21}NOF_2Si$) C, H, N.

Method B: Over a solution of **21b** (0.25 g, 1.25 mmol) in CH_2Cl_2 (3 mL) trimethylsilyl *N,N*-dimethylcarbamate (0.475 mL, 2.74 mmol) was added under argon. After 1.5 h, the solvent was evaporated, the residue taken in hexane, and the precipitate filtered off. The hexane was evaporated to dryness to afford **28b** (0.24 g, 70%) as an oil.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamate (29). Over a solution of **18b** (0.375 g, 1.0 mmol) in CH_2Cl_2 (15 mL) cooled to –78 °C, phosgene (20% in toluene, 0.75 mL, 1.5 mmol), 18-crown-6 ether (0.255 g, 1.0 mmol), and KF·HF (0.3 g, 3.8 mmol) were added. After stirring for 12 h and allowing the mixture to warm to room temperature, the fluoroformate **27** was formed (ν_{OCOF} 1827 cm^{-1}). The suspension was cooled to 0 °C, and a solution of **28a** (0.41 g, 1.35 mmol) in CH_2Cl_2 (5 mL) was added in one portion. After 1 h stirring, the suspension was filtered and the filtrate evaporated to dryness. The residue was purified by preparative HPLC (cyclohexane:AcOEt, 3:1) to afford **29** (0.31 g, 49%) as an oil. Mass ($C_{30}H_{35}N_2O_9Cl_2$) calcd, 637.1710; found, 637.1726. Anal. ($C_{30}H_{34}N_2O_9Cl_2$) C, H, N, Cl. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamic Acid (30).** **30** was obtained from **29** by the same method as **23** as a solid (0.105 g, 78.5%): mp 66–9 °C. Mass ($C_{24}H_{26}N_2O_9Cl_2Na$) calcd, 579.0913; found, 579.0903. Anal. ($C_{30}H_{35}N_3O_8Cl_2$) C, H; N: calcd, 5.04; found, 4.61. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-fluoroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamate (31). Method A: A solution of **18b** (0.4 g, 1.08 mmol) in THF (10 mL) and **27a** (prepared from 2.0 mmol of **21b**) was dissolved in THF (10 mL) and NEt_3 (0.25 mL, 1.8 mmol) added. The reaction mixture was stirred for 1.5 h and the solvent evaporated to dryness. The residue was purified by preparative HPLC (CH_2Cl_2:AcOEt, 39:1) to afford **31** (0.21 g, 35%) as an oil. Mass ($C_{30}H_{35}N_2O_9F_2$) calcd, 605.2311; found, 605.2330. Anal. ($C_{30}H_{34}N_2O_9F_2$): H, N; C: calcd, 59.60; found, 60.02.

Method B: Fluoroformate **27** (starting from 0.67 mmol of **18b**) was prepared as described for **29**. The suspension containing **27** was cooled at –78 °C, a solution of **28b** (0.12 g, 0.44 mmol) in CH_2Cl_2 (4 mL) added dropwise over 15 min, and the reaction mixture stirred for a further 30 min. The suspension was filtered, the filtrate evaporated to dryness, and the residue purified by preparative HPLC to afford **31** (0.018 g, 7%). ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-fluoroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamic Acid (32).** **32** (0.04 g, 42%) was obtained as an oil from **31** by the same method as for **23**. Mass ($C_{24}H_{26}N_2O_9F_2Na$) calcd, 547.1504, found, 547.1526. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamate (36). To a CH_2Cl_2 solution (3 mL) of **34** (0.147 g, 0.63 mmol) and triethylamine (180 mL, 1.25 mmol) was added triphosgene (63 mg, 0.21 mmol) in one portion. After 15 min stirring, the solvent was evaporated, the residue taken up in THF, and the insoluble salt filtered off. The THF was evaporated, and isocyanate **35a** (X = Cl) was obtained as an oil which was used immediately in the next reaction.

To the toluene solution (5 mL) of **18b** (60 mg, 0.15 mmol) was added isocyanate **35a** (X = Cl) (0.60 mmol) in toluene (5 mL) and dibutyltin dilaurate (10 mL). The reaction mixture was stirred for 12 h at room temperature, filtered, and evaporated to dryness. Purification was achieved by column chromatography (cyclohexane:AcOEt, 3:1), when product **36** (90 mg, 90%) was obtained as a solid: mp 44–7 °C; ν_{max}/cm^{-1} (film) 1728 (C=O, ester, carbamate). Mass ($C_{32}H_{43}N_3O_8Cl_2$) calcd, 667.2427; found, 667.2420. Anal. ($C_{32}H_{43}N_3O_8Cl_2$) C, H, N, Cl. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[*N*-(4-{bis[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamate (41) was obtained in a similar manner from **18a** and isocyanate **35b** (X = F) as a solid (0.205 g, 69%): mp 39–41 °C. Isocyanate **35b** (X = F) was prepared from **51** by the method previously described for **35a**. Mass ($C_{32}H_{43}N_3O_8F_2$) calcd, 635.3018; found, 635.3040. Anal. ($C_{32}H_{43}N_3O_8F_2$) H, N; C: calcd, 60.46; found, 60.03. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamic Acid (37).** Di-*tert*-butyl ester **36** (0.250 g, 0.37 mmol) was dissolved in formic acid (10 mL) and stirred under argon at room temperature for 48 h. The formic acid was evaporated under vacuum (oil pump) and the residue reevaporated again five times with CH_2Cl_2 and toluene to yield **37** as a solid (0.185 g, 89%): mp 99–102 °C; ν_{max}/cm^{-1} (film) 1713 (C=O, acid). Mass ($C_{24}H_{27}N_3O_8Cl_2Na$) calcd, 578.1073; found, 578.1070. Anal. ($C_{24}H_{27}N_3O_8Cl_2$) C, H, Cl; N: calcd, 7.57; found, 6.01. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[*N*-(4-{Bis[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamic acid (42)** was obtained identically from **41** as a solid (60 mg, 82%): mp 124–7 °C. Mass ($C_{24}H_{27}N_3O_8F_2$) calcd, 523.1766; found: 523.1780. Anal. ($C_{24}H_{27}N_3O_8F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)carbamoyl]-L-glutamate (39). The same procedure as for **36** was used starting from isocyanate **35a** (X = Cl) (0.54 mmol) in toluene (5 mL), linker **8b** (0.100 g, 0.27 mmol) dissolved in CH_2Cl_2 (5 mL), and dibutyltin dilaurate (10 mL). **39** was obtained as a solid. Further recrystallization from hexane:AcOEt (9:2) afforded **39** (0.110 mg, 64%) as a pure compound: mp 148–9 °C; ν_{max}/cm^{-1} (film) 1731 (C=O, ester), 1691 (C=O, carbamate), 1642 (C=O, urea). Mass ($C_{30}H_{36}N_4O_7Cl_2Na$) calcd, 657.1859;

found, 657.1850. Anal. ($C_{30}H_{36}N_4O_7Cl_2$) C, H, N, Cl. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

The same procedure was used to obtain **di-*tert*-butyl *N*-[(4-{[*N*-(4-{bis[2-chloroethyl]amino}phenyl)carbamoyloxy]-methyl}phenyl)carbamoyl]-L-glutamate (38)** (0.310 g, 95%), mp 68–71 °C, from **18a** and **35a**. Mass ($C_{32}H_{44}N_4O_7Cl_2Na$) calcd, 689.2485; found, 689.2480. Anal. ($C_{32}H_{44}N_4O_7Cl_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information. Also obtained was **diprop-2-enyl *N*-[(4-{[*N*-(4-{bis[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}-phenyl)carbamoyl]-L-glutamate (43)** (0.190 g, 75%), mp 152–4 °C, from **8b** and **35b**. Mass ($C_{30}H_{37}N_4O_7F_2$) calcd, 603.2630; found, 603.2650. Anal. ($C_{30}H_{36}N_4O_7F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)carbamoyl]-L-glutamic Acid (40).** Diallyl ester **39** (0.190 g, 0.3 mmol) dissolved in CH_2Cl_2 (5 mL) was stirred with $Pd(PPh_3)_4$ (15 mg) and pyrrolidine (120 mL, 1.45 mmol). The reaction mixture was kept under argon for 45 min and then diluted with AcOEt. The solvent was evaporated, and the insoluble residue was washed with AcOEt. It was dissolved in methanol (10 mL) and passed through an IRC50 ion-exchange column (weakly acid resin). After eluting with 50 mL of methanol, the eluate was evaporated under vacuum to yield **40** (0.100 g, 60%) as a solid: mp 127–9 °C. Mass ($C_{24}H_{28}N_4O_7Cl_2Na$) calcd, 577.1233; found, 577.1230. Anal. ($C_{24}H_{28}N_4O_7Cl_2$) H, N; C: calcd, 51.97; found, 51.54. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

The same procedure was used to obtain ***N*-[(4-{[*N*-(4-{bis-[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}-phenyl)carbamoyl]-L-glutamic acid (44)** from **43** as a glassy solid (0.125 g, 80%): mp 104–7 °C. Mass ($C_{24}H_{29}N_4O_7F_2$) calcd, 523.2004; found, 523.2020. Anal. ($C_{24}H_{28}N_4O_7F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

4-[Bis(2-fluoroethyl)amino]-*O*-benzylphenol (47). 4-[Bis-(2′-hydroxyethyl)amino]-*O*-benzylphenol (1.72 g, 6.0 mmol), triethylamine (4.3 mL, 31.0 mmol), and 4-(dimethylamino)-pyridine (0.120 g, 1.0 mmol) were dissolved in CH_2Cl_2 (40 mL) followed by methanesulfonic anhydride (4.3 g, 25.0 mmol) dissolved in CH_2Cl_2 (40 mL). After 1 h stirring the reaction mixture was extracted with aqueous citric acid 10% (100 mL) and the organic layer washed with water (100 mL), dried ($MgSO_4$), and evaporated to dryness. The residue was redissolved in acetonitrile (50 mL), KF (3.8 g, 65 mmol) and 18-crown-6 ether (0.5 g, 1.9 mmol) were added, and the mixture stirred at 60 °C for 48 h. The mixture was filtered and the filtrate evaporated to dryness. Purification was achieved by preparative HPLC (cyclohexane:CH_2Cl_2, 2:1) when **47** (1.0 g, 57%) was obtained as an oil: ^{1}H NMR δ_H 3.60 (2t, 4H, *N*-CH_2, J = 5.12 Hz, J_{H-F} = 22.5 Hz), 4.53 (2t, 4H, CH_2F, J_{H-F} = 47.5 Hz), 5.00 (s, 2H, $PhCH_2$-), 6.72 (d, 2H, $H_{arom2+6}$, J = 8.68 Hz), 6.87 (d, 2H, $H_{arom3+5}$), 7.30–7.45 (m, 5H, H_{arom}); MS *m/z* 291 (M^+, 100), 200 ($M^+ - PhCH_2$, 100). Mass ($C_{17}H_{19}F_2NO$) calcd, 291.1435; found, 291.1450. Anal. ($C_{17}H_{19}F_2NO$) C, H, N.

4-[Bis(2-fluoroethyl)amino]phenol (21b). 47 (1.0 g, 3.4 mmol) and pentamethylbenzene (3.8 g, 27.7 mmol) were dissolved in trifluoroacetic acid (25 mL) and stirred for 3 days at room temperature. TFA was then evaporated and the residue triturated with hexane. The insoluble oil was partitioned between acetonitrile (80 mL) and hexane (80 mL). The acetonitrile solution was separated, the solvent evaporated, and the residue taken in AcOEt (80 mL) and washed with aqueous $NaHCO_3$ (50 mL) and water (50 mL). The organic layer was dried ($MgSO_4$) and evaporated to dryness to yield a dark-yellow oil (0.670 g, 98%): ^{1}H NMR δ_H 3.54 (2t, 4H, *N*-CH_2, J = 5.25 Hz, J_{H-F} = 25.0 Hz), 4.51 (2t, 4H, CH_2F, J_{H-F} = 47.5 Hz), 6.64 (s, 4H, H_{arom}), 8.62 (s, 1H, OH); MS *m/z* 201 (M^+, 100), 182 ($M^+ - F$, 12). Mass ($C_{10}H_{13}F_2NO$) calcd, 201.0965; found, 201.0975. Anal. ($C_{10}H_{13}F_2NO$) C, H, N.

4-[Bis(2-fluoroethyl)amino]nitrobenzene (50). 4-[Bis-(2′-hydroxyethyl)amino]nitrobenzene (1.4 g, 6.2 mmol), triethylamine (4.3 mL, 31 mmol), and 4-(dimethylamino)pyridine (0.120 g, 1.0 mmol) were dissolved in CH_2Cl_2 (40 mL), and methanesulfonic anhydride (4.3 g, 25 mmol) dissolved in CH_2Cl_2 (40 mL) was added. After 1 h stirring the reaction mixture was extracted with aqueous citric acid 10% (100 mL) and the organic layer washed with water (100 mL), dried ($MgSO_4$), and evaporated to dryness. The residue was redissolved in chloroform (50 mL), Amberlyst 26 F^- was added, and the mixture was stirred at reflux for 72 h. The reaction mixture was filtered and the filtrate evaporated to dryness. Purification was achieved by preparative HPLC (cyclohexane:AcOEt, 3:1) when **50** was obtained as a solid. Recrystallization from hexane–diethyl ether–chloroform, 6:2:1, yielded **50** (0.290 g, 20%) as yellow crystals: ^{1}H NMR δ_H 3.87 (2t, 4H, *N*-CH_2, J = 5.1 Hz, J_{H-F} = 25.3 Hz), 4.64 (2t, 4H, CH_2F, J_{H-F} = 47.5 Hz), 6.92 (d, 2H, $H_{arom2+6}$, J = 9.48 Hz), 8.03 (d, 2H, $H_{arom3+5}$); MS *m/z* 231 (M^+ + 1, 100). Mass ($C_{10}H_{12}F_2N_2O_2$) calcd, 231.0945; found, 231.0955. Anal. ($C_{10}H_{12}F_2N_2O_2$) C, H, N.

4-[Bis(2-fluoroethyl)amino]aniline (51). 50 (0.260 g, 1.1 mmol) was dissolved in EtOH (12 mL); 10% Pd/C (0.120 g) and ammonium formate (0.500 g) were added. The suspension was stirred for 1.5 h at room temperature. The catalyst was filtered off and the filtrate evaporated to dryness. Purification was achieved by preparative HPLC (cyclohexane:AcOEt, 2:1) to yield **51** (0.190 g, 84%) as a dark-yellow oil: ^{1}H NMR δ_H 3.47 (2t, 4H, *N*-CH_2, J = 5.22 Hz, J_{H-F} = 25.0 Hz), 4.47 (2t, 4H, CH_2F, J_{H-H} = 47.5 Hz), 6.49 (d, 2H, $H_{arom2+6}$, J = 8.80 Hz), 6.60 (d, 2H, $H_{arom3+5}$); MS *m/z* 200 (M^+, 100). Mass ($C_{10}H_{14}N_2F_2$) calcd, 200.1115; found, 200.1130. Anal. ($C_{10}H_{14}N_2F_2$) C, H, N.

Chemical Stability Determination. Compounds were prepared as 10 mM concentrates in distilled water (**12**, **20**), DMSO (**23**, **25**, **37**, **40**, **42**, **44**), or MeOH (**30**) and diluted 100-fold in CPG2 assay buffer (100 mM Tris-HCl, pH 7.3; 260 μM $ZnCl_2$; 1 mL) to give 100 μM solutions. Aliquots (10 μL) were injected onto a Partisphere 5-μm C18 column (110 mm × 4.6 mm) and eluted isocratically (1 mL/min) with 10 mM ammonium acetate (pH 5.0) containing percentages of methanol chosen to produce a retention time of 3–4 min and monitored at the optimum wavelength (**12**, 10%, 246 nm; **20**, 10%, 213 nm; **23**, 63%, 255 nm; **25**, 50%, 250 nm; **30**, 65%, 258 nm; **32**, **37**, 58%, 268 nm; **40**, 58%, 253 nm; **42**, 45%, 267 nm; **44**, 43%, 251 nm). The amount of starting material remaining after various periods of incubation at 37 °C was determined either by repeat injections (7.5-min intervals) from a single vial (**12**, **20**, **30**, **32**, **37**, **40**, **42**, **44**) or by delayed injections (1-min intervals) from a new vial each time (**23**, **25**).

Kinetic Determinations. The optimum wavelength for spectrophotometric kinetic analysis was established from a differential spectrum comparing each parent compound with an equimolar mixture of its products (**12**, 250 nm; **20**, 228 nm; **40**, 280 nm; **42**, 267 nm; **44**, 251 nm). CPG2 (50–250 ng) was added to CPG2 assay buffer (100 mM Tris-HCl, pH 7.3; 260 μM $ZnCl_2$; 1 mL) containing 1–100 μM compound and the rate of change of absorbance at the chosen wavelength measured. In the case of the less stable **40**, compound was included in both reference and sample cuvettes to negate the contribution of purely chemical loss. Kinetic parameters were derived from Hanes–Woolfe plots.

Biological Methods: Cytotoxicity Assays. For this study, LS174T cells were constructed to express surface-tethered mutant CPG2 stably, by transfection of cells with the plasmid pMCEFstCPG2(Q)3[15] using lipofectamine, by the method previously described. The plasmid pMCEFstCPG2(Q)3 encodes for a CPG2 molecule that is expressed on the outer cell membrane. It bears three asparagine-to-glutamine [(Q)3] mutations that were otherwise inappropriately glycosylated. This plasmid encodes a mutated CPG2 molecule fused between the signal and the transmembrane domains of the c-erbB2 protein tyrosine kinase receptor under the transcriptional control of the EF-1a promoter and also encodes neomycin resistance for selection of stably expressing mammalian clones.

Single colonies were cloned by limiting dilution, and those expressing surface-tethered CPG2 were identified by their ability to degrade methotrexate[28] and by Western blot using a rabbit anti-CPG2 polyclonal serum. Control cells expressing β-galactosidase have already been described.[15]

The cytotoxicity of the compounds was assayed by a modification of the published procedure.[29] Cells (2×10^6) were seeded into 6-well plates, producing confluent monolayers in 48 h. Compounds were dissolved in DMSO at 10 mM (**30**) or 100 mM (**25**, **42**, **44**) immediately prior to treatment, diluted in full medium, and added to the wells. A similar concentration of drug solution was added after an incubation of 1 h, and the cells were incubated for an additional 20 h. The cells were harvested and reseeded in quadruplicate in 96-well plates at $\sim 2 \times 10^3$ cells/well and incubated until the control wells achieved confluence. The plates were fixed and stained with sulforhodamine-B, the extinction at 590 nm was read, the results are expressed as percentage of control growth, and the IC_{50} values were evaluated by interpolation.

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Supporting Information Available: Spectral data for the final compounds **12**, **20**, **23**, **25**, **30**, **32**, **37**, **40**, **42**, and **44**, the protected linkers **8a**,**b** and **18a**,**b**, the activated linkers **9a**,**b** and **19a**,**b**, the protected prodrugs **22**, **29**, **36**, **38**, and **39**, and the corresponding fluorinated analogues **24**, **31**, **41**, and **43** (16 pages). Ordering information is given on any current masthead page.

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